

CLINICAL APPLICATIONS OF THE NDOG2 MONOCLONAL  
ANTIBODY IN OVARIAN CANCER

JOHN ORRELL DAVIES

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This thesis is dedicated to my wife, Joanna.



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# UNIVERSITY OF EDINBURGH

## ABSTRACT OF THESIS (Regulation 7.9)

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Address .....

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NDOG2 is a murine IgG2b monoclonal antibody that recognises the three common allelic forms of placental alkaline phosphatase (PLAP) equally well. In addition NDOG2 recognises a second antigen of a similar molecular weight to the PLAP subunit (67,000 Daltons) which has no phosphatase activity.

The distribution of the NDOG2 determinant in normal tissue and in the common gynaecological malignancies was assessed using an indirect immunoperoxidase staining technique. Positive reactive staining was detected in normal fallopian tube, endometrial and endocervical columnar epithelium, thymus and lung as well as in term placentae. In addition positive staining was seen in 11 from 44 benign and in 36 from 56 malignant ovarian tumours. In both cases the serous histological type showed the strongest reactive staining and primary tumours showed a greater degree of staining than their secondaries. Similarly 11 from endometrial cancers and 5 from 12 cervical cancers showed reactive staining with NDOG2.

Two enzyme linked immunosorbent assays based on NDOG2 were developed. The first of these measured enzymically active PLAP and the second measured the total protein captured by NDOG2. 44 patients were followed up using both assays, 61% of whom either died of their disease or developed a significant recurrence during the time of the study. There was a close correlation between preoperative serum levels and the degree of reactive staining of the tumour in patients with ovarian cancer using both assays. Cigarette smoking was the major cause of raised serum PLAP in health and gave rise to a false diagnosis of tumour recurrence in one case. Serum levels of enzymically active PLAP predicted the course of the disease in 27% of cases studied whereas the second assay was of value in 45% of cases.

A 123 iodine labelled NDOG2 ( $^{123}\text{I}$  NDOG2) which retained over 90% of its specific activity after labelling, was used in the radioimmuno-detection of ovarian tumour deposits. Focal and diffuse abnormalities due to specific uptake of  $^{123}\text{I}$  NDOG2 by tumour were recognised and these correlated well with both the operative findings and the images obtained by ultrasound scanning and computer aided tomography. False positive results were most frequently due to masking of specific tumour uptake by areas of high background activity such as the liver and aorta. False positive results were generated by the subtraction techniques, designed to eliminate background activity, and by the presence of free  $^{123}\text{I}$  in gut mucosa and bladder.



STATEMENT BY CANDIDATE

The experimental work described in this thesis has been performed by myself, either independently or under the supervision of:

Dr P Jackson PhD

Dr B Randal PhD

Dr C Sunderland PhD

Professor G Stirrat FRCOG MD

unless otherwise stated in the text.

The NDOG2 antibody was produced by Sunderland, Stirrat and Redman in 1981. Financial support for this project was given by the South West Regional Health Authority, Xoma U.K. Ltd., and the Nichols Fellowship of the Royal Society of Medicine.

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Signed



## INTRODUCTION

Ovarian cancer is the most commonly occurring gynaecological malignancy in the U.K. (O.P.C.S. 1983) and because of its intra-abdominal spread presents problems, both in its early detection and in monitoring a patient's response to therapy. No reliable screening test exists for this disease but a number of methods have been employed to monitor the patient's response to therapy:

- 1) A 'second look' procedure (laparoscopy or laparotomy).
- 2) Biochemical serum markers.
- 3) An imaging technique such as ultrasound scanning (U.S.S.), Xray computed tomography (C.T.) or radioimmunoscintigraphy (R.I.S.).

Second look procedures whilst giving accurate information about the state of the disease have a morbidity and mortality not associated with the other two groups. In addition a second look laparotomy can appreciably reduce the quality of life remaining for those patients with residual disease. The other two methods, while relatively free of side effects, do give false positive and negative results. C.T. in particular, but also U.S.S., suffers from relatively poor sensitivity and specificity when applied to small, solid intra-abdominal tumour deposits, after primary surgery. In the search for an improved imaging technique antibodies have been used to target radionuclides to tumour deposits (radioimmunoscintigraphy). Obviously the choice of antibody is crucial. The first ideal would be an antibody that recognises a totally tumour specific antigen without any cross reactivity with normal tissues.

The second ideal would be an antibody which recognises only one epitope. This is achieved by monoclonal antibodies which provide a single antibody preparation with constant specificity. This contrasts with polyclonal antisera which can cross react unpredictably with a variety of tissues (Lennox and Sikora 1982). This constant specificity gives monoclonal antibodies theoretical advantages over polyclonal antisera when used as the basis for serum assays to monitor a particular biochemical serum tumour marker.

Unfortunately, in the case of epithelial ovarian cancer no truly tumour specific antigen exists although over 40 tumour associated antigens have been described (Umbach 1984). Placental alkaline phosphatase (P.L.A.P.) is one such antigen, and has been reported in association with a variety of tumours including ovarian carcinoma. Apart from term placentae P.L.A.P. has been reported in small quantities in the epithelium of the upper female genital tract, thymus, lung and testis (Goldstein et al 1982, Sunderland et al 1984, Davies et al 1985). However, in the case of ovarian cancer, production of P.L.A.P. by placenta and testis is obviously irrelevant and as hysterectomy is usually performed during primary surgery this source of the enzyme is effectively removed. Production by the thymus is minute and it is the lung that potentially offers the greatest problem in the use of this antigen in monitoring patients with this disease.

This thesis describes the use of a murine monoclonal antibody, designated NDOG2, which recognises the three common allelic forms of placental alkaline phosphatase (P.L.A.P.) in ovarian cancer. NDOG2 has been used in three ways:

- a) To demonstrate immunohistologically the distribution of P.L.A.P. in normal adult organs and its presence on ovarian and certain other gynaecological tumours. This is described in Chapter 3.
- b) As the basis of a sensitive enzyme linked immunosorbant serum assay (E.L.I.S.A.) to assess the role of P.L.A.P. as a biochemical marker in ovarian cancer. The development of various assays and the predictive value of P.L.A.P. in these tumours is described in Chapter 4.
- c) 123-iodine ( $^{123}\text{I}$ ) labelled NDOG2 has been used in the radioimmuno-detection of both primary, metastatic and recurrent ovarian cancer and this is described in Chapter 5.

CHAPTER ONE

OVARIAN CANCER, TUMOUR MARKERS AND  
PLACENTAL ALKALINE PHOSPHATASE

### 1.1. Introduction

This chapter describes the incidence, aetiology and pathology of ovarian cancer and discusses the problems in management of patients with this disease. Various biochemical tumour markers in ovarian cancer are described and one such marker, placental alkaline phosphatase (PLAP) is discussed in detail. It is acknowledged that at least two isoenzymes of alkaline phosphatase are present in the human placenta but the term PLAP, as used in this thesis, refers to the heat stable, L-phenylalanine inhibitable isoenzyme.

### 1.2. Ovarian Carcinoma

#### 1.2.1. Incidence and Mortality

The incidence of ovarian carcinoma has been increasing this century and it is now the most commonly occurring gynaecological cancer in England and Wales. Overall it is the sixth commonest cancer in women (Table 1.1.) with 4,317 new cases reported in 1979, giving an incidence of 17.1/100,000 (OPCS Monitor 1983). This incidence is increasing in women 65 years of age and over, is constant between the ages of 45 and 64 and falling in the 16 to 44 year age group (Cancer Statistics 1981). Chart 1.1. and Table 1.2. show the age related incidence for ovarian cancer (all types) in 1979.

Ovarian carcinomas alone are responsible for more deaths than cervical and endometrial carcinomas together and only cancers of pancreas, lung and stomach have a poorer prognosis (Cutler 1976).

### 1.2.2 Aetiology

There are several theories as to the aetiology of ovarian carcinoma:

#### a) "Incessant Ovulation"

The term "incessant ovulation" was first coined by Fathalla (1971). He postulated that each ovulation caused minor trauma to the serosal (epithelial) coat of the ovary which is then exposed to high levels of oestrogen from the follicular fluid. Stein and Allen (1942) had already shown, in animal experiments, that oestrogens injected into the ovarian capsule causes proliferation of the epithelium.

Zajicek (1978) suggested that the trauma caused by ovulation led to inclusions of surface epithelium in the stroma of the ovary and that these could develop into ovarian cysts and carcinomas.

Therefore, one would expect anything that reduces the number of ovulations to protect against the subsequent development of ovarian carcinoma. Thus, there is an inverse relationship between the number of pregnancies and ovarian carcinoma (Joly et al 1974, Beral et al 1978, Cassagrande et al 1979), and the oral contraceptive has also been shown to be protective (Cassagrande et al 1979).

This theory could explain the present increase in incidence of ovarian carcinoma i.e. the population at present at risk of developing ovarian carcinoma tended to have smaller families, to bottle feed their infants and used non hormonal methods of contraception.

b) Talc

Talcum powder is similar to, and until recently was frequently contaminated by asbestos, a known carcinogen for serosal epithelium. It has been suggested that talc can migrate to the ovary via the cervical canal, uterine cavity and fallopian tube from the vagina or vulva where it has been introduced by condoms, caps or deodorants (Venter and Harralde 1979) 75% of ovarian carcinomas were shown to contain talc particles in one series (Henderson et al 1971) although no ovarian carcinoma has ever been induced using talc in animal experiments.

c) Oestrogens

Oestrogens have been shown to cause ovarian epithelial hyperplasia in animals (Stein and Allen 1942) and the association between breast, endometrial and ovarian carcinomas have led some workers to implicate oestrogens as a causative factor (Wynder et al 1969). In addition obesity, which is known to cause hyperoestrogenism by the conversion of androstenedione to oestrone by adipose tissue (Edman and McDonald 1978), is associated with an increased risk of the development of ovarian carcinoma (Cassagrande et al 1979).

d) Family History

In a few instances a strong family history can be obtained for ovarian carcinoma (Luraine and Piver 1979, Franceschi et al 1982, Piver 1983).



e) Viral Infections

Childhood viral infections such as mumps, measles, chickenpox and rubella are thought to be protective against ovarian cancer (Newhouse et al 1972, West 1966) but this may simply be due to a secondary effect on ovulation rather than a protective effect of the virus itself (Newhouse et al 1972). However, there is an increased risk of ovarian carcinoma in patients who have developed rubella infection between the ages of 12 and 18 when compared with those who developed the illness before the menarche (McGowan et al 1979).

f) Coffee

Heavy coffee drinkers have been shown to have a slightly increased risk of ovarian carcinoma (Hartage 1982). However, this is unlikely to be a major cause of ovarian carcinoma worldwide.

g) Association with other conditions

Carcinoma of the ovary can be associated with certain inherited conditions such as Peutz-Jeghers syndrome (familial intestinal polyposis) (Dozois et al 1970). These tumours are usually granulosa cell tumours and have a distinctive pattern suggesting that a mutant gene is responsible for both conditions (Scully 1970).

h) Conclusions

The true aetiology of ovarian cancer is uncertain, however, ovulation with the formation of inclusions of surface epithelium in the stroma of the ovary gives a starting point for the development of ovarian epithelial tumours. However, not every woman who ovulates develops ovarian cancer and the protective effect of pregnancy is out of

all proportion to the number of ovulations prevented. It is likely, therefore, that there is some external carcinogen. Talc has been a favourite candidate for many years, although the fact that it does not induce ovarian cancers in animal models must cast doubt on its role. Possible contamination with asbestos may have caused some ovarian cancers in the past. It should also be noted that talc while chemically similar to asbestos exists as rounded and not pointed particles, and so does not have "asbestos" irritant effect on epithelial cells. The effect of oestrogen, unopposed by progestogens, on the inclusion cysts would seem to be a more likely cause for malignant change, but the evidence for this is not conclusive.

#### 1.2.3 Normal embryological development of the gynaecological organs

The fallopian tube, uterus and cervix all arise from the paramesonephric (Mullerian) duct. This structure appears as an invagination of the coelomic mesothelium into the mesenchyme on the lateral surface of the upper part of the urogenital ridge at six weeks gestation. The paramesonephric ducts are bilateral and, as they pass caudally, fuse to form the primitive uterus and cervix, the unfused portions forming the fallopian tubes. The walls of these structures are formed from the surrounding mesenchyme but the epithelium of the fallopian tube, endometrium and cervical columnar epithelium is derived from the mullerian duct epithelium (Janowski and Paramanandhan 1973).

The caudal tip of the fused mullerian ducts will form the upper two-thirds of the vagina; the lower third and vulva are formed from the urogenital sinus (Craigmyle and Presley 1979).

The ovaries arise as an area of thickening of the mesoderm of the genital ridges and are covered by a layer of coelomic mesothelium known as the germinal epithelium (Willis 1958). This latter is a misnomer as the germ cells arise from the area of the yolk sac and migrate to the ovary. The germinal epithelium becomes the serosa of the ovary and the ovarian stroma develops from the mesoderm of the genital ridges (Craigmyle and Thomas 1979).

Thus, the ovarian serosa, fallopian tube, endometrial and cervical columnar epithelium all arise from the same area embryologically and are all of mesodermal origin, whereas the cervical and vaginal squamous epithelium is thought to migrate cephalad from the urogenital sinus and are ectodermal in origin.

#### 1.2.4 Pathology, mode of metastases and presentation of epithelial ovarian cancer

Epithelial ovarian carcinoma make up 80-90% of all ovarian malignancies. Sex cord/stroma and germ cell tumours make up the remaining 10-20% but these tumour groups tend to respond well to therapy and it is the epithelial cancers that contribute to the high mortality associated with this disease.

Epithelial tumours are so called because they arise from the serosal coat of the ovary which itself is derived from the germinal epithelium (Janovski and Paramanandhan 1973). There are three common types of epithelial ovarian tumours. Serous tumours have a cell type similar to fallopian tube epithelium, mucinous tumour cells are similar to cervical columnar epithelium and endometrioid tumour cells have a similar pattern to that of endometrium.

Ovarian cancer spreads predominantly by the shedding of cells by the primary tumour. The pumping action of the diaphragm causes free floating tumour cells to move from the pelvis through the peritoneal cavity with a particular propensity for collecting in the omentum (Pemberton 1940) and right subdiaphragmatic area (the Leinorenal ligament tends to stop cells reaching the left hemidiaphragm) (Bagley and Piver 1983).

Lymphatic spread to the pelvic (Musumeci 199a) and aortic nodes (Knapp and Friedman 1974) is relatively common and in the latter case can be present in those patients who would otherwise be classified as having Stage 1 disease only.

Bloodborne spread is mainly to liver and lung, occurring by definition in only late (Stage 4) disease.

Because of this propensity for intra-abdominal metastasis a patient with ovarian carcinoma frequently presents with advanced disease, the symptoms of early and even moderately advanced disease tending to be non-specific and vague. The most common presenting symptoms are abdominal distension and bloating (44%) non-specific digestive abnormalities (38%) pelvic discomfort (33%) and abnormal vaginal bleeding (13%) (Piver 1976). Many of these symptoms e.g. obvious abdominal distension are only present with advanced disease.

#### 1.2.5 Problems in the management of patients with ovarian cancer

##### i) Screening

The ideal way of managing patients with this disease is to diagnose the tumour before it has metastasised. A number of screening tests have been devised:

a) Routine vaginal examination

It has been estimated that only 1 in 10,000 tumours will be detected in this manner and it is sobering to realise that a tumour of 1cm diameter would contain in the region of 1 billion cancer cells. Tumours of this size are virtually impossible to palpate. 95% of ovarian malignancies however are 5cm or more in diameter and the finding of an ovarian mass of this size warrants further investigation, particularly if there is also a contralateral ovarian mass.

b) Culdocentesis

The instillation, aspiration and cytological examination of saline from the pouch of Douglas has been proposed as a screening test for this disease (Funkhaiser et al 1975) but the results have been disappointing and the procedure is unacceptable to a large number of women.

c) Imaging techniques

Ultrasound scanning (U.S.S.) and computerised axial tomography (C.T.) have both been postulated as imaging techniques.

C.T. is expensive (C.T. scanners cost upwards of £500,000), time consuming, carries a radiation hazard and presents certain difficulties in interpretation of images both in the obese and very thin patient. In addition previous pelvic surgery can also confuse the issue. U.S.S. is less time consuming and does not have the other disadvantages of C.T., but will not detect solid masses of less than 2cm diameter. In addition, the differentiation between benign ovarian tumours, loops of bowel, hydrosalpinges and endometriosis from ovarian cancer is difficult, depending very much on the skill of the individual operator.

Radioimmunoscentigraphy offers potential in the detection of small intra-abdominal tumours and this will be discussed later in this thesis.

d) Laparoscopy and Laparotomy

These are unacceptable screening methods but have a role in the management of patients with established ovarian cancer.

ii) Management

The ideal scheme of treatment for a patient with epithelial ovarian cancer includes:

a) A thorough and adequate staging procedure (the FIGO classification for staging ovarian carcinomas is summarised in Table 1.3). This will involve, where appropriate, cytological examination of any ascites or of peritoneal washings and scrapings, from the subdiaphragm; histological examination of omentum and, if there is no obvious extra ovarian spread, of peritoneal biopsies and of the para-aortic nodes.

b) Cytoreductive surgery, including total abdominal hysterectomy, bilateral salpingo-oophorectomy and omentectomy, aiming to remove if possible all macroscopic tumour. In the event that this is not feasible then to leave deposits of less than 2cm diameter (Piver 1983).

c) Chemotherapy or radiotherapy for all patients with greater than Stage 1a(i) tumours.

The problem then arises as to how to monitor the patient's response to therapy. Ovarian cancer predominately metastasises intra-abdominally and it is difficult to detect small tumour deposits by physical examination alone. Standard imaging techniques such as C.T. and U.S.S. have limitations when applied to small solid intra-abdominal tumour deposits. Therefore a 'second look' procedure is performed, in some centres, on patients who are apparently in remission after a course of treatment and in whom a decision needs to be made about the need for further chemotherapy.

Over the last 10-20 years possible biochemical tumour markers, and more recently, radioimmunoscintigraphy have been investigated to try and obviate the need for a second look operation in these patients.

#### 1.2.6 Tumour Markers in Epithelial Ovarian Cancer

A tumour marker can be defined as any identifiable change in a body component indicative of the presence of cancer. Thus abnormal papanicolaou smears could be considered to be tumour markers for cervical carcinoma. In the case of ovarian cancers biochemical tumour markers have been used. An ideal marker would have to be produced by the ovarian tumour in its earliest growth phase in quantities to be easily measurable, would have a short biological half life, decrease with successful treatment and would accurately predict recurrence before this was clinically demonstrable. Although in gynaecological practice Beta subunit H.C.G. and alphafoetoprotein have proved useful markers in trophoblastic neoplasia and embryonal



sinus tumours respectively, no similar markers exist for epithelial ovarian cancer. Indeed over 40 potential tumour markers have been described (Umbach 1984) but none have proved particularly useful in the management of this disease.

Tumour markers can be divided into carcinoplacental fetal or tumour associated antigens, and miscellaneous tumour markers. P.L.A.P. is, in this classification, a carcinoplacental antigen, but it will be considered separately after describing other tumour markers most frequently used in the management of patients with ovarian cancer.

#### 1) Carcinoplacental Antigens:

##### Human Chorionic Gonadotrophin (H.C.G.)

This was first recognised as a tumour marker for trophoblastic disease in 1956 (Coppelson 1981) and it is in this group of tumours that it still has its greatest use. H.C.G. has proved useful in the management of some ovarian germ cell tumours such as choriocarcinoma, embryonal carcinoma and some malignant teratomas (Newlands 1982, Piver 1983). In epithelial ovarian cancers it has variously been reported in association with 5-50% of cases (Stone et al 1977, Morrow and Townsend 1981, Coppelson 1981). It has been reported as being most common in serous cystadenocarcinoma (Morrow and Townsend 1981) but levels of H.C.G. only appear elevated in patients with gross disease (Barker 1980) and rapidly return to normal after the start of therapy even when there is marked residual tumour (Stone et al 1977).



### Human Placental Lactogen (H.P.L.)

H.P.L. has been described in association with both germ cell and epithelial ovarian cancers (Caspar et al 1984). However, although up to 75% of patients with primary epithelial ovarian cancers have elevated serum H.P.L. this serum marker does not rise with early recurrent disease (Samaan et al 1976) but can become elevated in end stage disease (Crowther et al 1979).

### Placental Proteins

Alpha macroglobulin has been reported to be elevated in the sera of a large percentage of ovarian cancer patients by some workers (Malkin et al 1978, Sawada et al 1982) but others (Damber et al 1976) have failed to detect it in these patients.

Raised serum B-1 glycoprotein levels have been reported only in a small percentage of patients with advanced epithelial ovarian carcinomas (Tatarinov 1978, Crowther et al 1979).

The placental specific proteins numbered 5 and 11 have been demonstrated in association with a large number of ovarian cancers but their clinical value has yet to be evaluated (Coppleson 1981, Inaba et al 1982).

### Bjorkland Isoenzyme or Tissue Polyseptide Antigen (T.P.A.)

T.P.A., a polyseptide of 22,500 molecular weight, is found in normal placentae and in association with 46% of cancers of all types including ovary. Transient raised serum levels are noted in 22% of patients with infectious diseases, 25% of patients with non malignant diseases and 5% of the normal population (Bjorkland 1976). Its value as a tumour marker in ovarian cancer has not been evaluated.

## Fetal Antigens

### 1) Carcinoembryonic Antigen (C.E.A.)

This membrane bound glycoprotein was initially reported in association with adenocarcinoma of the colon (Gold and Freeman 1965). It has subsequently been found in association with a variety of cancers including ovary (Khoo and McKay 1974) but is also raised in a variety of non malignant diseases and in cigarette smokers (Hansen et al 1974). It has proven to be a very popular tumour marker for ovarian carcinomas with an association reported between 13.4% (Stone et al 1977) and 65% (Khoo et al 1979). C.E.A. has been variously reported as being most commonly elevated in association with both serous cystadenocarcinomas and poorly differentiated tumours (Khoo and McKay 1976) and also with mucinous cystadenocarcinomas (Morrow and Townsend 1981, Coppleson 1981). The incidence of elevated C.E.A. levels has been shown to rise with advancing stage of the disease (Khoo and McKay 1974, Di Saia et al 1975, Van Nagell et al 1975) but some workers (Barker 1980) suggest that it is only elevated in patients who are in the terminal stages of their disease. Conversely Khoo and McKay (1976) suggest that C.E.A. falls during the terminal illness.

All these workers have used polyclonal antisera to C.E.A. as a basis for their assays, which will therefore recognise a number of different antigenic determinants. As C.E.A. and a variety of other normally occurring glycoproteins e.g. biliary glycoprotein and a glycoprotein found in the washings of normal colon, share common epitopes (Lennox and Sikora 1982) it is not surprising that there is such a variation in results obtained from one group of workers to another.

Khoo and McKay (1976) found C.E.A. to be a useful tumour marker for "gynaecological malignancies" predicting tumour recurrence by up to 10 weeks before it was detectable clinically, but false positives and negatives have been reported (Khoo et al 1979, Caspar et al 1984). Other workers have not shared this enthusiasm (Stone et al 1977, Barker 1980, Samaan et al 1976) perhaps reflecting the problems in using polyclonal antisera alluded to earlier.

## 2) Alpha Foetoprotein (A.F.P.)

This is the main foetal plasma protein and its first reported tumour association was with hepatoma (Abelev 1963). It is a good tumour marker for ovarian endodermal sinus tumours (Sell et al 1976) and is also found in embryonal cell carcinomas. A.F.P. is additionally associated with other germ cell tumours containing yolk sac elements (Van Nagell et al 1981) but it is not a reliable marker in this last group. However, A.F.P. is only rarely found in association with epithelial ovarian carcinoma (Stanhope et al 1979, Smith and Oi 1984).

## 3) Other Foetal Antigens

Alpha 1 antitrypsin, transferrin, prealbumin, foetal ferritin, Beta oncofoetoprotein and foetal sulphoglycoprotein have all been described in association, to varying degrees with ovarian cancers, but have not proved to be of use in this disease (Piver 1983, Smith and Oi 1984).

### Tumour Associated Antigens

Tumour associated antigens are predominantly membrane bound glycoproteins, which are, at least in theory, only found in association with malignancies; however, as is discussed below, this is not always the case.

#### 1) Ovarian Cystadenocarcinoma associated antigen (O.C.A.A.)

This antigen is found in association with both serous and mucinous cystadenocarcinomas as well as carcinomas of breast, cervix and colon and has not so far been detected in normal adult tissue (Bhattacharya and Barlow 1973, 1975, 1979). It is elevated in the sera of 66% of patients with Stage 2 and 3 disease and 80% of patients with Stage 4 disease but was not detected in the single patient with Stage 1 disease (Piver et al 1979) however in 70% of these patients O.C.A.A. accurately predicted the clinical course of the disease (Bhattacharya and Barlow 1979).

#### 2) Ovarian Cancer Antigen (O.C.A.)

O.C.A. is found at all stages of the disease in the sera of 65% of patients with serous or mucinous cystadenocarcinomas and in 54% of those with endometrial tumours. It is also found in 10% of normal controls (Knauf and Urbach 1978, 1980). A longitudinal study is required to assess its usefulness in clinical practice.

#### 3) C.A. 125

This antigen was originally thought to be totally specific for ovarian carcinoma (Bast et al 1981), it has since however been detected in normal adult endometrium, fallopian tube epithelium, cervical columnar epithelium and in association

with non-specific inflammation (Kabawat et al 1983, Niloff et al 1984). It now appears that all carcinomas arising from tissues derived from the mullerian duct also express this antigen (Niloff et al 1984). 82% of patients with ovarian epithelial carcinoma have raised serum levels and in 93% of these serial serum levels of C.A. 125 proved to be accurate in predicting the course of the disease, serum C.A. 125 is also elevated in 6% of the population. Interestingly C.A. 125 has many properties similar to P.L.A.P. in both its distribution in gynaecological malignancies, normal tissue and structure. However, it is a distinct antigen (Davies et al 1985, Bast et al 1985).

#### 4) Carbohydrate Antigen C.A. 50

This antigen was initially recognised in colorectal adenocarcinoma cells. It has subsequently been found in a variety of epithelial tumours including 40% of epithelial ovarian carcinomas (advanced Stage 3 and 4 tumours only), (Holmgren et al 1984). Its value as a tumour marker has yet to be decided.

#### 5) Human Milk Fat Globule 2 (H.M.F.G.2) Antigen

This antigen is found in all normal secretory epithelium but is present in much higher concentrations in certain cancers, including ovarian carcinoma. Immunohistological examination of ovarian cancer tissue, using the H.M.F.G.2 monoclonal antibody, has shown heterogeneity of expression of this antibody within individual tumours (Hammersmith Oncology Group 1984). A radioimmunoassay has demonstrated elevated levels of H.M.F.G.2 antigen in the serum of 53% of patients with advanced breast cancer, in five from six

patients with active ovarian cancer but also in 16.6% of control sera (Burchell et al 1984). The H.M.F.G.2 antibody has also been successfully used in radioimmunoscintigraphy (see p. 80-81).

## MISCELLANEOUS

### 1) Hormones

#### Luteinising Hormone

This hormone has proven to be of no clinical use in the management of epithelial cancers (Stanhope et al 1979).

#### Oestrogens

These are only of use in monitoring patients with granulosa cell tumours (Aakvaag 1975).

#### A.C.T.H., A.D.H., Parathormone

These hormones have a role in monitoring patients with that extremely rare group of hormone producing tumours (Norris 1975, Muggia et al 1975).

### 2) Enzymes

#### Lactate Dehydrogenase (L.D.H.)

Cancer cells have increased glycolysis and so patients with carcinoma should have a raised serum level of this enzyme. L.D.H. is therefore a non-specific tumour marker and some workers have found that a high proportion of ovarian cancer patients have raised serum levels (Awais 1973 and 1978). Burrows (1980) however failed to detect any significant increase in serum levels of this enzyme in cancer patients when compared to controls. L.D.H. has also been reported in association with dysgerminoma (Friedman et al 1984) and endodermal sinus tumour (Takeuchi et al 1979).

### Glycosyltransferases

The glycosylation of proteins is different in cancer cells than in normal cells (Rostenberg et al 1978). The glycosyltransferase, galactosyltransferase, has been shown to be a good tumour marker by some, being elevated in all cases of ovarian carcinoma and predicting recurrence of the disease up to 7 months before it was clinically obvious (Chatterjee et al 1980). Other workers have found elevated galactosyltransferase levels in between 32 and 64% of patients with ovarian tumours (Gaudichon et al 1983, Waalkes 1982).

### Alpha-L-fucosidase

Alpha-L-fucosidase is involved in the metabolic pathway of the deoxyhexose, L fucose (6 deoxyl-L-galactose). This sugar is widely distributed in human tissues and is an important component of the oligosaccharides responsible for blood group specificity. Although levels of alpha-L-fucosidase have been described as being higher in ovarian cancer tissue when compared to normal ovary (Chatterjee et al 1979), serum levels of this enzyme are significantly reduced in patients with ovarian cancer (Bhattacharya and Barlow 1979). The reasons for this are unclear and further work is needed to ascribe a role for this enzyme, as a tumour marker, in patients with this disease.

### Plasminogen Activator

This enzyme is released by ovarian tumour cells in culture (Swanberg and Antedt 1976) but has not found a use in clinical practice.



### 3) Others

#### Urinary Hydroxproline

This is a breakdown product of collagen and has been detected in raised amounts in urine of patients with advanced disease or metastases, sometimes before clinical recognition (Coppleson 1981).

#### Fibrin Degradation Products (F.D.P.) and Erythrocyte Sedimentation Rate (E.S.R.)

Anstey and Blythe (1978) demonstrated elevated F.D.P. in virtually 100% of patients with ovarian cancer. However both F.D.P. and E.S.R. are elevated in a large number of not necessarily malignant conditions and therefore cannot be considered to be useful tumour markers.

#### Cyclic Guanosine 3'5' Phosphate (Cyclic G.M.P.)

Serum levels of cyclic G.M.P. have been reported to be elevated in 90% of patients with ovarian cancer, but is also elevated in a variety of other conditions (Turner et al 1980).

### 1.3 Placental Alkaline Phosphatase

I shall consider the structure, function, intracellular site and methods for distinguishing between the different isoenzymes of P.L.A.P. before discussing it's distribution in both health and disease.

#### 1.3.1 The structure of Placental Alkaline Phosphatase

Placental alkaline phosphatase (P.L.A.P.) is a dimer with each subunit having a molecular weight of 74,000 Daltons (Stinson and Seargeant 1981). Alkaline phosphatase is a glycoprotein; carbohydrate, mainly glucosamine galactosamine,



hexosamine and sialic acid, makes up 15-30%, by weight, of the molecule (McComb 1979) and there are approximately 1000 aminoacids per subunit (Nozawa and Fishman 1982). The configuration of aminoacids at the active site however varies between the different isoenzymes. In the case of P.L.A.P. it is:

$P_1$

Thr-gly-Pro-Asx-Val-Thr-Anx-Ser-Ala-Ala-Ser-Ala-His-glx-glx-leu.

Phosphate binds, as shown, to the first serine molecule (Whittaker et al 1976).

The P.L.A.P. molecule contains 4 zinc atoms, two of which are structural and hence firmly bound, the other two atoms are loosely bound and are needed for catalytic activity (Fishman 1974).

A high molecular weight (750,000 Daltons) form of P.L.A.P. has recently been described (Ahu Hassan et al 1984). So far this has only been detected in term placentae.

### 1.3.2 Reactions catalysed by P.L.A.P.

All alkaline phosphatase isoenzymes catalyse the same reactions namely hydrolysis and phosphate transfer (Gutman 1959). This enzyme catalyses the hydrolysis of the P-O-C bond (Gutman 1959), P-F bond (Fernley and Walker 1967), P-O-P bond (Fernley and Bisaz 1968), P-S bond (Neuman 1968) and the P-N bond (Fernley 1971) of a variety of monophosphate esters to yield inorganic phosphate and the corresponding sugar, alcohol or phenol etc.. The reaction proceeds in two phases (1) after the formation of the enzyme-substrate complex, hydrolysis of the substrate generates a phosphoryl-enzyme and product, (2) the phosphoryl-enzyme dissociates to give inorganic phosphate and enzyme (Fishman 1974).

Alkaline phosphatase has been thought to be the same as two other enzymes, namely  $\text{Ca}^{2+}$  ATPase and inorganic pyrophosphatase. Although ATP is a substrate for both enzymes (Eaton 1967, Moss 1969) Fishman (1974) demonstrated by aminoacid inhibition, that they are different enzymes. Inorganic pyrophosphatase, while sharing many properties with alkaline phosphatase, is inhibited whereas the latter is activated by magnesium (Cathala and Brunel 1973).

#### 1.3.3 Intracellular site of P.L.A.P.

Alkaline phosphatase activity has been demonstrated by electron microscopy, on the plasma membrane lining the microvilli in kidney proximal tubule (Goldfisher et al 1964), intestine (Hugon and Borghers 1966) and term placental syncytiotrophoblast (Hulstaert et al 1973), in this latter case P.L.A.P. was also demonstrated on the basal surface of the syncytiotrophoblast. Tokumitsu et al 1981, demonstrated alkaline phosphatase intracellularly in a variety of non malignant cells, associated predominantly with the endoplasmic reticulum, golgi apparatus, mitochondrion and in the perinuclear space. Both the cytoplasmic and membrane alkaline phosphatases had identical properties suggesting the former is simply en route from its site of production to the cell membrane. A similar situation has been reported in ovarian tumours (Johnson et al 1984).

#### 1.3.4 The function of P.L.A.P.

A precise role has yet to be attributed to any of the isoenzymes of alkaline phosphatase. However, as they are located, in the main, in organs involved in absorption and/or excretion and are membrane bound, they may well

participate in the active transportation of substances across the cell membrane (Kabat and Furth 1940, McComb 1979). A role for alkaline phosphatase in the regulation of cell membrane dimensions was postulated by Fishman and Lin (1973). Nozawa and Fishman (1982) finding phosphoryl histone products to be a substrate for this enzyme, suggest a role in gene regulation.

#### 1.3.5 Differentiation between the various isoenzymes of alkaline phosphatase

There are three isoenzymes of alkaline phosphatase. The alkaline phosphatase found in liver, bone, kidney and placenta before 10 weeks gestation have many similar properties and are referred to as the tissue non-specific isoenzyme (Nozawa and Fishman 1982). Alkaline phosphatase present in intestine and term placentae, respectively make up the other two isoenzymes.

Isoenzymes are a group of enzymes having the same function but different structures, it is therefore possible to distinguish between them using immunological methods. The various isoenzymes also have different physical and biochemical properties and these are discussed below:

##### a) Urea and Heat Stability

There is a close association between inactivation of the various isoenzymes of alkaline phosphatase by urea and heat (Fennelly et al 1969).

The placental isoenzyme is the most heat stable and resistant to urea denaturation followed by the intestinal and tissue non-specific isoenzymes (Birkett et al 1967, Butterworth and Moss 1967) (Table 1.4).

Heat stability varies with pH; both the intestinal and tissue non-specific enzymes being most stable at pH 7 (Moss et al 1972).

b) Specific Inhibitors

A variety of substances, mainly aminoacids, have been described that selectively inhibit the action of one or more isoenzymes. Bodansky (1939) was the first to use a specific inhibitor (bile acids) to distinguish between tissue non-specific and intestinal isoenzymes. Since then a variety of inhibitors have been described including L-phenylalanine (Ghosh and Fishman 1966 and 1968, Fernley and Walker 1970), L-Leucine (Nakayama 1970, Wei and Doellgast 1981), L-tryptophan (Lin and Fishman 1971), L-homoarginine (Lin and Fishman 1972), Levamisole (Van Belle 1972, Borgers 1973), L-Leucyl-glycyl-glycine and L-phenylglycyl-glycine (Nozawa and Fishman 1982); these inhibitors are only effective as the L-isomer and their effect on the various isoenzymes of alkaline phosphatase is summarised in Table 1.5.

The mechanism of inhibition has been postulated as either

1) the formation of a poor dissociable enzyme-inhibitor-substrate complex (Fishman and Ghosh 1967); or

2) preventing the breakdown of the phosphoryl-enzyme intermediate formed at the end of the first phase of the reaction to enzyme and inorganic phosphate (Fernley and Walker 1969, Byers et al 1972).

### Effect of pH

Alkaline phosphatase is distinguishable by its high (alkaline) pH optimum from acid phosphatase. At pH 6 the alkaline phosphatase starts to split into monomers due to the dissociation of zinc, by pH 4 all the enzyme is monomeric (Applebury 1969). This trend can be reversed at pH 5-6 by the addition of excess zinc or cobalt, but below pH 5 the enzyme cannot be reactivated (Harkness 1968).

The various isoenzymes have different pH optima:

	Tissue non-specific	Intestinal	Placental
pH optima	10.1-10.2	10.1-10.2	10.7

However, all the isoenzymes are active at physiological pH.

### Electrophoretic Mobility

P.L.A.P. was first distinguished as a separate isoenzyme using starch gel electrophoresis (Boyer 1961). Prior to this the high levels of alkaline phosphatase in pregnancy serum were thought to derive from fetal or maternal bone or maternal liver. P.L.A.P. was found to occupy an intermediate position between the tissue non-specific (fast) and intestinal (slow) isoenzymes on electrophoretic migration. Since 1961 a number of other media support have been used in electrophoresis i.e. cellulose acetate membrane, polycrylamide and agar gells. These media have various properties and have all been used to differentiate the various isoenzymes of alkaline phosphatase (Nozawa and Fishman 1982).

In order to overcome problems with diffuse or overlapping bands of the various isoenzymes some workers have used specific inhibitors, urea denaturation or heat to exclude one or more isoenzyme (Fennelly 1969, Green et al 1972). Alternatively the molecular weight and negative charge of the placental and tissue non-specific isoenzymes can be reduced by splitting sialic acid from the molecule, using neuraminidase, which will therefore separate these from the intestinal isoenzyme which does not contain sialic acid (Fishman 1974).

Electrophoresis has also been used to distinguish between the various forms and variants of P.L.A.P., starch gel proving to be the most useful matrix in this respect (Inglish et al 1973, Fishman 1974). The more recent technique of isoelectric focusing on a polyacrylamide gel has also proved useful (Stinson and Seargeant 1981).

#### Affinity Chromatography

This is a method primarily to purify proteins but can also be used to distinguish between the various isoenzymes of alkaline phosphatase using their variable lectin binding affinity, the intestinal isoenzyme is totally unbound, whereas the tissue non-specific isoenzyme shows variable binding (Trepanier et al 1976, Nozawa and Fishman 1982).

#### Immunology

The three isoenzymes of alkaline phosphatase were first recognised to be antigenically distinct by Boyer in 1963, but whilst antisera raised against the tissue non-specific isoenzyme does not cross react with either of the other two



isoenzymes (Sussman et al 1968), both intestinal and placental alkaline phosphatase appear to share at least one epitope (Doellgast et al 1976, Lehman et al 1976). This similarity causes difficulties in distinguishing between these two isoenzymes using polyclonal antisera even after appropriate absorption procedures (Haiji et al 1979).

Monoclonal antibodies, which recognise a single antigenic site, will reliably distinguish between P.L.A.P. and intestinal alkaline phosphatase provided the site recognised is not shared and a number of such antibodies have been described (Slaughter et al 1981, Millan et al 1982 a & b, McLaughlin et al 1983, Sunderland et al 1984).

#### SUMMARY

P.L.A.P. is a heat stable isoenzyme of alkaline phosphatase which is resistant to urea denaturation and is characterised by its inhibition by the L-isomers of phenylalanine, tryptophane, levamisole, phenylglycylglycine and leucylglycylglycine. It has a high optimal pH of 10.7, intermediate electrophoretic mobility and a high lectin binding affinity. However, the difference between the various isoenzymes is often only one of degree. P.L.A.P. and intestinal alkaline phosphatase in particular share many physical and biochemical properties as well as having antigenic similarities. This can lead to difficulties in distinguishing between these two isoenzymes particularly where one is present at a much higher concentration than the other. Monoclonal antibodies to P.L.A.P. have proven to be very sensitive tools in the detection of P.L.A.P.. Their practical applications are discussed in Chapter 2.

### 1.3.6 Evolution of P.L.A.P.

It is clear from their physical, biochemical and immunological properties that there are three alkaline phosphatase isoenzymes i.e. tissue non-specific (liver, kidney, bone), intestinal and placental. Further evidence for this classification comes from the study of the amino acid sequence of the various isoenzymes. Liver, bone and kidney alkaline phosphatases all have an identical peptide map which is distinct from those of the other two isoenzymes (Seargeant and Stinson 1979). Therefore, as all three groups have a different structure, they must be coded for by three separate genes, one for the tissue non-specific isoenzyme, one for intestinal alkaline phosphatase and one for P.L.A.P..

By studying the immunological properties of alkaline phosphatase from various animal groups a plan of the evolution of the three human isoenzymes can be postulated (fig 1.2). An enzyme identical to the human tissue non-specific isoenzyme is found in fish, amphibians, reptiles, birds and mammals (Goldstein and Harris 1979, Nozawa and Fishman 1982) suggesting that this enzyme is an early evolutionary product. The intestinal isoenzyme is only found in mammals (Nozawa and Fishman 1982) and shares close immunological properties with P.L.A.P. suggesting a common origin (Lehman 1976). An enzyme with close properties to P.L.A.P. first appears in higher mammals and certain primates, but is less heat stable and L-phenylalanine sensitive (Rogers 1982); "Human" P.L.A.P. is only found in man, chimpanzee and the orang-utan (Doellgast et al 1979).



### 1.37 Types of P.L.A.P. in Health and Disease

#### a) In Human Placenta

At present P.L.A.P. is the most polymorphic human enzyme known. P.L.A.P. was first recognised as a distinct enzyme by Boyer (1961) who was also able to identify four phenotypes which he named A, AB, B and D in descending order of electrophoretic mobility. He also suggested that these three common phenotypes might be genetically determined as their frequencies in the population were such as would be predicted by the Hardy Weinberg Law on a 1 allele hypothesis.

Robson and Harris (1965) showed that human P.L.A.P. classified into at least six phenotypes based on electrophoretic mobility at varying pH's. These they called F, FI, I, SI, FS and S. They postulated that Boyer's type 'A' phenotype was composed of the F, FI and I forms, the 'AB' phenotype was composed of the FS and SI forms and the 'B' phenotype was equivalent to the S form.

They suggested that there were three alleles coding for these forms and that they were a mixture of homozygotes (F, S, I) and heterozygotes (FI, SI, FS), (Table 1.6). The homozygous form was found to have one component on electrophoresis and the heterozygote had three components, two equivalent to the corresponding homozygotes and the third in an intermediate position equivalent to a hybrid substance. These six phenotypes are known as the common forms and are found in 98% of human placentae. However in the remaining 2% the P.L.A.P. expressed is a rare variant, the slow moving D band in Boyer's original paper is thought to be composed of a number of rare variants. Harris et al (1967) identified nine rare

variants which were classified according to their electrophoretic mobilities. They postulated that there needed to be three common and six rare alleles at an autosomal locus to code for these forms. The potential combination of common forms and rare variants from a total of nine alleles would equal 35 (six common, 29 rare). Donald and Robson (1974) looked at 5,000 placentae and discovered a further 10 of these rare variants but also found an additional 19 new rare variants whose phenotype was such that they could not be coded for by the original six rare alleles. They concluded that a total of 18 alleles (three common, 15 rare) were needed to include all the phenotypes found. This gives a possible total of 126 different forms and variants, however only six common forms and 38 rare variants have been described. In view of the large number of alleles present the old F, S, I nomenclature was abandoned in favour of a numerical classification where the alleles are numbered 1-18. Thus, S is equivalent to 1, F = 2, I = 3 and so on, this remains the standard method for classifying the various forms and variants of P.L.A.P. present in human placentae. There are, however, an increasing number of monoclonal antibodies recognising different epitopes of P.L.A.P. being produced (Slaughter et al 1981, Millan et al 1982a, McLaughlin et al 1983) and these are beginning to be used to distinguish between the different types of P.L.A.P. present both in malignant and non-malignant tissues (Slaughter et al 1983, Millan and Stigbrand 1983, McLaughlin and Johnson 1984, Sunderland 1984).

b) In Malignant and Non-Malignant Tumours

At present the classification of the various forms of P.L.A.P. found in tissues other than placenta is confused and is undoubtedly an over-simplification of the true state of affairs. Four different forms of alkaline phosphatase have been described in association with various malignancies, usually taking their name from the patient in whom they were first recognised, of these four only three have some P.L.A.P. like characteristics, the fourth the non-regan isoenzyme has identical properties to the isoenzyme found in early pregnancy (i.e. the tissue non-specific form) it will be described for completeness but will not be referred to again.

1) Regan Isoenzyme

This isoenzyme was originally found in association with a lung carcinoma in a patient called Peter Regan, from whom it takes its name (W H Fishman 1968). The regan isoenzyme has identical electrophoretic mobility to the F phenotype of P.L.A.P., is sensitive to L-phenylalanine, resistant to L-homoarginine inhibition, has identical heat stability, enzyme kinetics and immunological properties as P.L.A.P. (Stolbach et al 1969). Further evidence that the regan isoenzyme is similar to the common forms of P.L.A.P. comes from a study of the subunit molecular weight, isoelectric points, aminoterminal sequences and peptide mapping (Greene and Sussman 1973).

2) Nagao Isoenzyme

This isoenzyme was first reported in a patient (Mr Nagao) with pleuritis carcinomatosa. It has the same immunological properties and the same heat and urea stability as the

common forms of P.L.A.P.; however it is markedly more sensitive to both L-Leucine and ethyl-enediamine tetra-acetic acid (E.D.T.A.) and is slightly more sensitive to L-phenylalanine. The nagao iso-enzyme has a much slower electrophoretic mobility than the S, I and F phenotypes and this coupled with its L-Leucine sensitivity has led some workers to suggest that the nagao isoenzyme is the re-expression of the rare 'D' variant of P.L.A.P. by cancer cells (Inglis et al 1973). However, Doellgast and Wei (1981) identified three immunoreactive sites on the 'D' variant and nagao isoenzyme, only one of which was common to both, suggesting a close relationship but different structure.

### 3) Warnock variant (Regan variant, Kasahara variant)

This variant was first identified in a group of patients with hepatocellular carcinoma and is heat stable and L-phenylalanine sensitive with a faster electrophoretic mobility than either the liver or placental isoenzyme (Warnock and Reisman 1969). It has been thoroughly investigated and found to share properties with both P.L.A.P. (i.e. L-phenylalanine and neuraminidase sensitivity, L-homoarginine resistant, urea stability, molecular weight and antigenicity) and the liver isoenzyme (i.e. pH optimum and phosphate sensitivity). It differed from both in its electrophoretic mobility, L-Leucine and E.D.T.A. sensitivity and was marginally less heat stable than P.L.A.P. (Higashino et al 1972).

It is probable that this variant is a hybrid isoenzyme composed of subunits of P.L.A.P. and intestinal alkaline phosphatase as the Warnock variant reacts with antisera against P.L.A.P. and the intestinal isoenzyme but not liver alkaline phosphatase (Higashino et al 1974 & 1975).

### Non Regan Isoenzyme

The majority of alkaline phosphatase secreting tumours produce this enzyme (Nozawa and Fishman 1982); it was first described in a patient with lung cancer (Timperley 1968) and is identical to the isoenzyme found in human placentae from 6-10 weeks gestation (L Fishman et al 1976, Sakyam et al 1979).

The properties of these various isoenzymes are summarised in Table 1.7.

### 1.3.8 Expression of P.L.A.P. in Health

For many years P.L.A.P. was thought to be found only in the serum of pregnant women. This view changed with the discovery of a P.L.A.P. like enzyme in the serum of a patient with lung carcinoma (Fishman et al 1968). Stolbach et al (1966) and Usategui-Gomez (1974) found P.L.A.P. in the serum of healthy non-pregnant individuals and Fishman and Nathanson (1971) found P.L.A.P. in the serum of patients with blood group O blood.

P.L.A.P. has been described in:

#### a) Placenta

Placental alkaline phosphatase first appears in pregnancy serum at about 10 weeks gestation and shows an exponential rise to a peak at 37-40 weeks gestation (Fishman et al 1972). The serum levels of P.L.A.P. havenot, however, been shown to be of use in the detection of placental insufficiency (Fishman et al 1972, Marshall and Parisi 1975, McLaughlin et al 1983). After 16 weeks gestation all the alkaline phosphatase in the human placenta is of

the placental isoenzyme (Nozawa and Fishman 1982) and is found on the outer cell membrane of the syncytiotrophoblast covering the chorionic villi, (Dempo et al 1979). There is no P.L.A.P. in the villous stroma (Dempo et al 1979).

P.L.A.P. is found in one of its six common forms in 98% of placentae and as one of at least 38 rare variants in the remaining 2% (Donald and Robson 1974).

b) Endometrium

Several workers have reported trace amounts of P.L.A.P. in normal endometrial epithelium (Malkin et al 1979, Nozawa et al 1980). 21.9% of cases showed P.L.A.P. in surface epithelium using histochemical methods based on heat stability and aminoacid inhibition (Nozawa et al 1980). Other workers however (Goldstein et al 1980) have not detected P.L.A.P. in endometrium.

c) Cervix

Malkin et al (1979), Nozawa et al (1980) and Goldstein et al (1980 and 1982) have all detected P.L.A.P. in cervical columnar epithelium. Goldstein et al (1980) estimated that 18% of the alkaline phosphatase activity in cervical columnar epithelium and mucus was due to P.L.A.P.. The isoenzyme has properties similar to the 'Regan isoenzyme' and is L-Leucine resistant (Goldstein 1982). However, small quantities of an L-Leucine sensitive form of P.L.A.P. have also been detected in normal cervix (McLaughlin et al 1984).



d) Testis

Chang et al (1978) and Goldstein et al (1982) have both demonstrated P.L.A.P. in normal testis. This isoenzyme was shown to be L-Leucine sensitive and it was postulated that this was the 'Nagao isoenzyme', (Goldstein et al 1982). McLaughlin et al (1984) have confirmed this finding but also detected trace quantities of one of the common forms of P.L.A.P.

e) Thymus

The form of P.L.A.P. found in this organ was also L-Leucine sensitive (Goldstein 1982).

f) Lung

Trace quantities of an L-Leucine resistant form of P.L.A.P. have been detected in normal lung (Goldstein et al 1982) as well as varying amounts of an L-Leucine sensitive form (McLaughlin et al 1984). It is interesting to note that raised serum P.L.A.P. of up to ten times the normal, non-smoking range has been recorded in cigarette smokers. The levels did not correlate with the number of cigarettes smoked a day (Maslow et al 1983, Tonik et al 1983). However, a form of P.L.A.P. recognised by one monoclonal antibody (McLaughlin et al 1983) does not appear to be present in the serum of smokers, although this antibody does recognise P.L.A.P. present in normal lung (McLaughlin et al 1984).

### 1.3.9 Expression of P.L.A.P. in Disease

#### a) Non malignant disease associated with elevated serum P.L.A.P.

Nathanson and Fishman (1971) reported raised serum P.L.A.P. in patients with non malignant as well as malignant disease. They used heat stability and polyclonal antisera to differentiate between P.L.A.P. and the intestinal and tissue non-specific isoenzymes. Elevated serum P.L.A.P. was found in:

1) Alcoholic cirrhosis	2 from 8 cases
2) Ulcerative colitis	2 from 4 cases
3) Diverticulitis	1 from 1 case
4) Peripheral vascular disease	3 from 7 cases
5) Hydronephrosis	1 from 2 cases

Cigarette smoking at that time was not recognised as a cause of raised P.L.A.P. and so this might explain some or all of the elevated levels (particularly those with peripheral vascular disease) and cross reactivity between placental and intestinal isoenzymes may explain elevated levels in those patients with intestinal disorders.

#### b) P.L.A.P. and Malignancy

Although P.L.A.P. has been described in association with a large number of tumours (Table 1.8) it should be noted that cigarette smoking, now recognised as a cause of elevated P.L.A.P. (Maslow et al 1982) was not taken into account in all of these papers.



P.L.A.P. has been used as a tumour marker with varying degrees of success. Thus, although raised serum P.L.A.P. has been described in association with breast carcinoma in between 14% and 21% of cases (Nathanson and Fishman 1971, Cadeau et al 1974 and Wada et al 1979) McDicken et al (1983) could not detect elevated serum P.L.A.P. in cases where immunohistology of the tumours had been positive (7 positive out of 7 cases). This latter finding was confirmed by McLaughlin et al (1983). Non Regan isoenzyme has also been demonstrated in association with this tumour (Miyayama et al 1976). It is possible that although the tumour type produces P.L.A.P. (as shown histologically), the serum levels are too low to detect (McDicken et al 1983). The elevated serum levels in early studies could all have been due to cigarette smoking.

However, in the case of seminoma, P.L.A.P. has been demonstrated immunohistologically on resected specimens (Wahren et al 1979) and approximately 60% of such patients with active disease have elevated P.L.A.P. levels (Lange et al 1982). Other workers have found P.L.A.P. to be a useful tumour marker in this group of patients (Jepsson et al 1983).

#### 1.3.10 P.L.A.P. in Genital Cancer

##### a) Ovarian Carcinoma

P.L.A.P. was first recognised in association with ovarian cancer by Stolbach et al in 1969. This finding has subsequently been confirmed by many workers, often without distinguishing the form of P.L.A.P. expressed. Between 22% and 39% of patients with ovarian cancer

have elevated serum levels of this enzyme (Nathanson and Fishman 1971, Stolbach et al 1976, Malkin et al 1978, Van Nagell 1981). Fishman et al (1975) used inhibition with L-Leucine to distinguish between the types of P.L.A.P. present and found that while ascitic fluid P.L.A.P. was, in 82% of cases, the L-Leucine sensitive form, this was only present in serum in 35% of these cases. An L-Leucine sensitive form of P.L.A.P. was also detected in tumour tissue extracts but again this was not necessarily reflected in serum P.L.A.P. levels (Benham et al 1979).

Various groups (Cadeau et al 1974, Kellen et al 1976 and Haije et al 1979) have found a P.L.A.P. like enzyme to accurately reflect tumour load. Haije et al using an enzyme linked immunosorbant assay based on polyclonal antisera, stress the usefulness of 'carcinoplacental alkaline phosphatase' as a tumour marker, finding it elevated in 40% of patients, predominantly those with serous and endometrioid cystadenocarcinomas and that it accurately predicted the course of the disease in 86% of patients. McLaughlin et al (1983) have also found elevated serum P.L.A.P. using an enzyme immunoassay based on a monoclonal antibody, in 35% of patients with ovarian cancer. However, Doellgast and Holmesley (1984) while finding elevated serum P.L.A.P. in patients with ovarian cancer did not find it to be a useful tumour marker although they found that a high preoperative level suggested a poor prognosis. Burrows (1980) failed to detect significantly raised serum P.L.A.P. in any patient with ovarian cancer.

P.L.A.P. has been used as part of a 'battery' of other tumour markers and although there is an increased pick up rate for at least one marker, the practical value of this mode of follow up is questionable (Fishman et al 1975, Stolbach et al 1976).

b) Cervical Carcinoma

Nathanson and Fishman (1971) found raised serum P.L.A.P. in association with this tumour in 22% of cases and this finding has been confirmed by others (Van Nagell 1981). Nonetheless P.L.A.P. would not appear to be of use as a tumour marker in this type of cancer. Cadeau et al (1974) found 17% of patients with active disease to have raised serum levels but also found elevated levels in 25% of cases who were in remission and other workers found that the incidence of raised serum P.L.A.P. is in indirect proportion to the stage of the tumour (Kellen et al 1976, Malkin et al 1978).

c) Endometrial Cancer

Cadeau et al (1974) found elevated P.L.A.P. in the sera of 12% of patients with endometrial carcinoma. Van Nagell et al (1981) confirmed this observation but some workers have failed to detect raised serum P.L.A.P. in such patients (Nathanson and Fishman 1971). Variable amounts of P.L.A.P. were found in endometrial carcinoma tissue extracts in 29 from 33 cases (Doellgast and Holmesley 1984) and Nozawa et al (1981) histochemically demonstrated P.L.A.P. in 7 from 42 cases of this tumour.

As with cervical carcinoma P.L.A.P. has not been thought to be a useful tumour marker here, Kellen et al (1976) only found elevated serum P.L.A.P. levels in Stage Ia tumour and could not detect it in the serum of any patient with later stage disease.

d) Seminoma Tumour of the Testis

Wahren et al (1979) detected elevated serum P.L.A.P. in the preoperative serum samples in 10 out of 19 patients with active seminoma. Similar figures were obtained in two subsequent larger studies (Lange et al 1982, Jeppsson et al 1983). In all three studies serial serum P.L.A.P. measurements were of value in monitoring the course of the disease in between 60% and 75% of patients studied. None of these studies considered the effect of cigarette smoking in elevating serum P.L.A.P. (Maslow et al 1983) and it is of interest to note that in two of these studies between 13% (Jeppsson et al 1983) and 35% (Lange et al 1982) of patients in complete remission also had elevated serum P.L.A.P. levels.

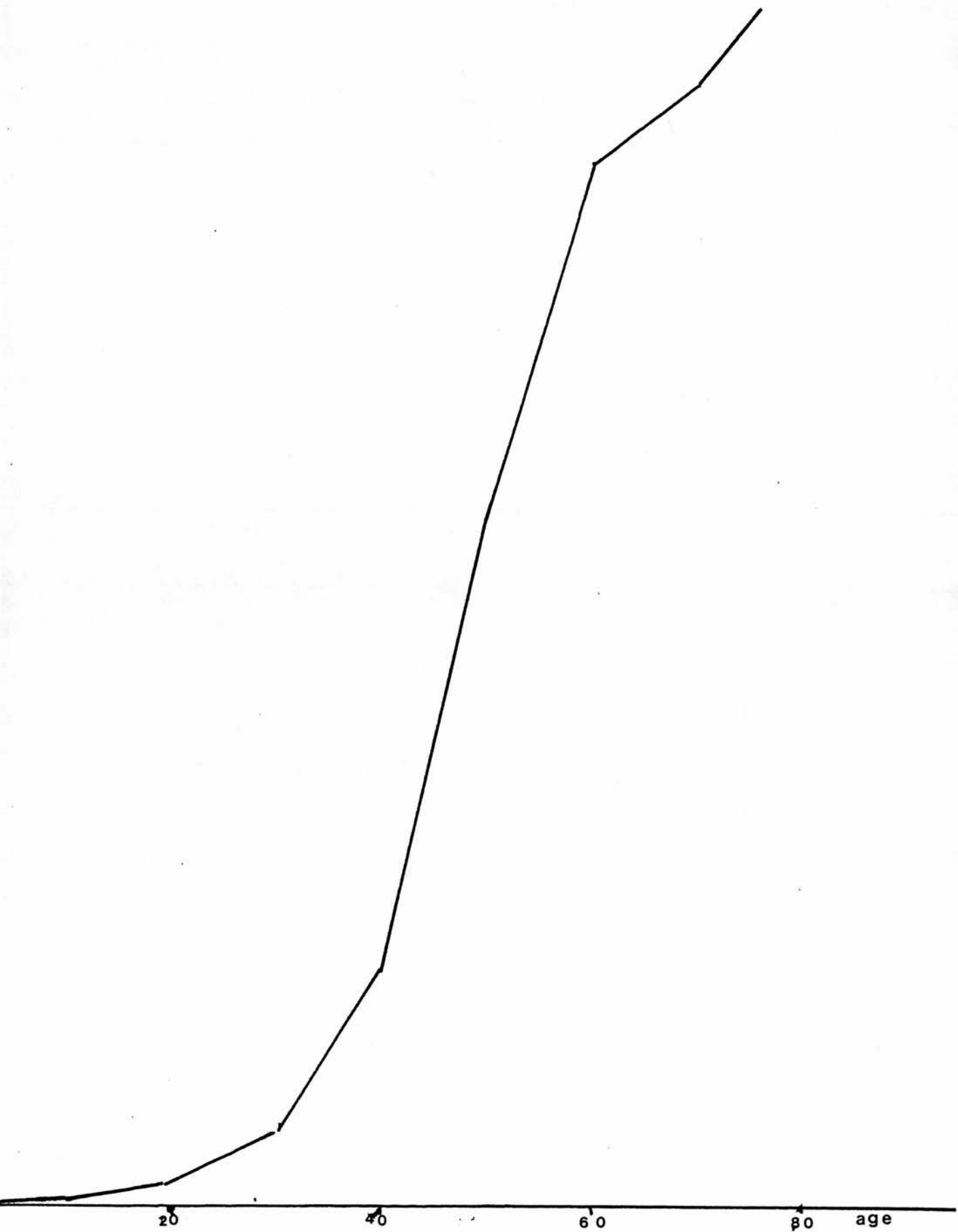


Fig 1.2    The Geneology of P.L.A.P.

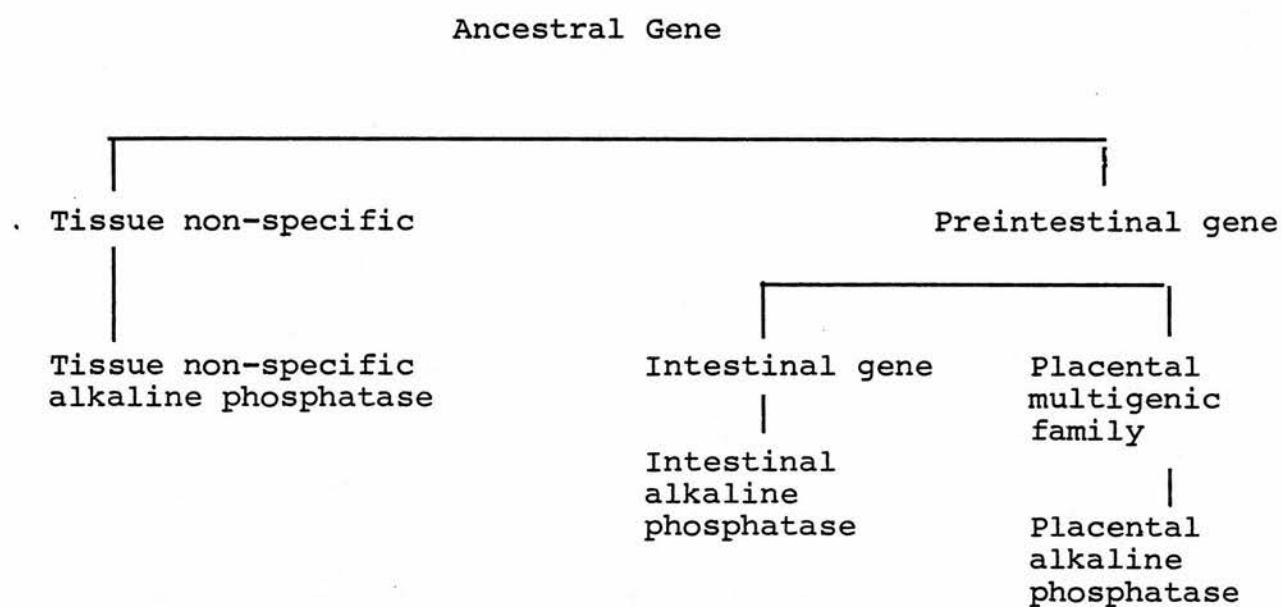


TABLE 1.1.NUMBERS OF NEW CASES OF THE TEN COMMONEST FEMALE  
CANCERS (1979)

Cancer Type	Number	Percentage of whole
Breast	20,648	22%
Skin (excl. melanoma)	9,154	10%
Trachea, Bronchus, Lung	8,341	9%
Colon	7,877	9%
Stomach	4,903	5%
Ovary	4,317	5%
Rectum & Anus	4,182	5%
Cervix	3,879	4%
Endometrium	3,466	4%
Pancreas	2,814	3%

Figures for carcinoma of the cervix excludes in-situ lesions.

TABLE 1.2INCIDENCE OF OVARIAN CARCINOMA PER 100,000 WOMEN BY  
AGE (1979)

<u>AGE</u>	<u>INCIDENCE PER 100,000</u>
0-4	0.1
5-14	0.3
15-24	0.9
25-34	2.8
35-44	9.1
45-54	26.8
55-64	40.4
65-74	43.6
75 <sup>+</sup>	46.2



Table 1.3 F.I.G.O. Stage-grouping for primary carcinoma of the ovary

<u>Stage 1</u>	Growth limited to the ovaries
1a	Growth limited to one ovary; no ascites
	(i) No tumour on external surface, capsule intact
	(ii) Tumour on external surface and/or capsule breached
1b	Growth limited to both ovaries; no ascites
	(i) & (ii) as for Stage 1a
1c	Growth limited to one or both ovaries with malignant ascites or cells in peritoneal washings
<u>Stage 2</u>	Growth invading one or both ovaries with pelvic extension
2a	Extension and/or metastases to uterus and/or tubes
2b	Extension to other pelvic organs
2c	Stage 2a or 2b tumour with malignant ascites or cells in peritoneal washings
<u>Stage 3</u>	Growth involving one or both ovaries with intra-peritoneal metastases outside the pelvis and/or retroperitoneal nodes. Metastasis to the surface of the liver is also Stage 3 disease.
<u>Stage 4</u>	Growth involving one or both ovaries with distant metastases. If pleural effusion is present there must be positive cytology to allot a case to Stage 4. Parenchymal liver metastases equals Stage 4.



Table 1.4 The effect of heat and urea on the isoenzymes of alkaline phosphatase

Isoenzyme	Heat stability		Urea Denaturation
	15 min at 56°C	15 min at 65°C	
Tissue non-specific	+ / ++	-	+++
Intestinal	++	-	++
Placental	+++	+++	+ / -

- = None

+ - +++ = Degree of stability/denaturation

Table 1.5 Inhibitors (I)<sub>50</sub> mmol/L of the 3 standard A.L.P. types

	Tissue non-specific	Intestinal	Placental
L-phenylalanine	31	0.8	1.1
L-homoarginine	2.8	40	>50.0
L-phenylalanyl glycylglycine	30.6	3.7	0.1
Levamisole	0.03	6.8	1.7
L-Leucine	13.1	3.6	5.7

(from Goldstein et al 1982)

TABLE 1.6

PHENOTYPE, GENOTYPE AND ELECTROPHORECTIC MOTILITY OF THE  
6 COMMON FORMS OF P.L.A.P.

Phenotype	Electrophoretic Motility	Genotype
SS	Slow	$pl^s$ $pl^s$
SF	Intermediate	$pl^s$ $pl^f$
SI	Intermediate	$pl^s$ $pl^i$
II	Intermediate	$pl^i$ $pl^i$
FI	Fast	$pl^f$ $pl^i$
FF	Fast	$pl^f$ $pl^f$

Table 1.7 Properties of tumour associated alkaline phosphatase (Nozawa 1982)

DEVELOPMENTAL COUNTERPART	Non-Regan	Warnock	Regan	Nagao
	Early fetal placenta from 6-10 weeks gestation	Hybrid to iso- enzyme composed of intestinal and D-variant subunits	Term placenta (10-16 weeks onwards) (S, I, F)	Term placenta rare variant similar to 'D' variant
pH optima	10.1	10.1	10.6	10.6
Heat stability -56°C for 30 min -65°C for 5 min	- -	+ -	+++ +++	+++ +++
Aminoacid L-phenylalanine	+	+++	+++	+++
Sensitivity L-homoarginine L-leucine	+++ +	+ ++	+ +	+ +++
Electrophoretic Migration	Fast	Fast	Intermediate	Intermediate
Neuraminidase Sensitivity	+	+	+	+
Reaction with antisera to: Liver A.L.P.	+	-	-	-
Intestinal A.L.P.	-	++	+	+
Term placental A.L.P.	-	+	+++	+++

Table 1.8      P.L.A.P. Producing Tumours

<u>Tumour</u>	<u>% Expressing P.L.A.P.</u>	<u>Reference</u>
* Adrenal Cortex	33%	1
* Breast	14%	1
Bile Duct	100% (1 patient)	2
Cervix	14%	1
* Colon	10%	1
* Hepatoma	80%	3 & 4
* Kidney	7%	1
* Lung	17%	1
* Lymphoma	9%	1
Mesothelioma	33%	1
* Ovary	20%	1
* Pancreas	20%	1
Prostate	14%	1
* Sarcoma	11%	1
Seminoma	100% (1 patient)	1
* Seminoma	60%	5
* Stomach	15%	1

\* Refers to tumour type with 10 or more cases studied.

#### References

- 1 = Nathanson and Fishman    1971
- 2 = Jacoby and Bagshawe      1971
- 3 = Warnock and Reisman      1969
- 4 = Higashino et al            1972
- 5 = Lange et al                1982

## CHAPTER TWO

### MONOCLONAL ANTIBODIES

## 2.1 Introduction

A monoclonal antibody is a defined chemical entity recognising a single antigenic determinant, which can be reliably and easily reproduced. Polyclonal antisera however, are undefined mixtures of antibodies which recognise a variety of epitopes and whose composition varies from batch to batch produced. The production of such antisera requires prolonged animal immunisation schedules and time consuming purification procedures whereas monoclonal cell lines may be grown up simply in cell culture.

Any antibody, be it monoclonal or derived from polyclonal antisera, can potentially recognise a number of different antigens, provided they all carry the relevant epitope. However, whereas cross reactivity can be accurately predicted in the case of a monoclonal antibody, this is not so in a variable mixture of antibodies. Unfortunately, this very purity of a monoclonal antibody may prevent it from recognising its antigen should a change of environment (e.g. formalin fixation) alter its epitope, a situation less likely to occur with polyclonal antisera which recognise many epitopes and are therefore partially self correcting.

This chapter describes the theory of production and discusses the potential uses of monoclonal antibodies with particular reference to the clinical applications of the NDOG2 antibody used in this thesis.

## 2.2 Production of Monoclonal Antibodies

### 2.2.1 Theory of production of monoclonal antibodies

The basis of the monoclonal technique is that one B-lymphocyte, in response to a given antigenic stimulus, will only produce one antibody. If this lymphocyte can be cloned then the resultant cell line will produce a pure antibody of single specificity. Unfortunately, lymphocytes will not survive for long in cell culture. However, myeloma cell lines will grow in culture and although there are many that will produce an antibody of their own there are some that are non secretors e.g. the N.S.1. cell line. The method of fusing a lymphocyte with such a myeloma cell line was first described by Kohler and Milstein in 1975 and the resultant tetraploid hybrid cell is able to grow in cell culture medium and will produce a single antibody.

The myeloma cell lines used have certain genetic deficiencies which do not allow them to grow under certain conditions. These cell lines are unable to incorporate externally supplied hypoxanthine or thymidine as they lack the appropriate salvage pathway. Thus, when the endogenous synthesis of D.N.A. precursors is blocked with methotrexate or aminopterin, then these cells die even in the presence of hypoxanthine or thymidine. Lymphoid spleen cells do possess the salvage pathway but will not survive in cell culture. Therefore only the hybrid cells will survive in a culture medium containing H.A.T. (hypoxanthine, aminopterin and thymidine), the growth of these cells is encouraged by the presence of non fused spleen cells referred to as feeder cells.



The hybrid cells are individually isolated by dilution so that the resultant colonies are all derived from a single cell i.e. are clones. The steps in the production of monoclonal antibodies are summarised in fig 2.1 (Milstein 1982).

#### 2.2.2 Problems in the production of monoclonal antibodies (Bastin et al 1982)

These usually stem from infection of one sort or another. Bacterial, yeast or fungal infections are usually obvious and are due to imperfect aseptic technique, contamination of cell culture media or from fungal spores in the atmosphere. Mycoplasma infections on the other hand are more insidious, often presenting as failure of the hybrid colonies to grow or the cessation of antibody production. Mycoplasma may be introduced with the myeloma cell line which can continue to grow, albeit more slowly, while infected.

Some clones grow poorly in low cell density cultures, the addition of extra feeder cells and 2 mercapto-ethanol ( $10^{-4}$ M) + 20% fetal cell serum helps to overcome this problem.

Overgrowth by non secreting clones will cause the cessation of antibody production by hybrid cells. The culture should, therefore, have been cloned earlier.

#### 2.2.3 Production of monoclonal antibodies to trophoblast antigens

The NDOG2 cell line was produced by Sunderland et al in 1981 and it is from this cell line that all the NDOG2 antibody used in the experiments in this thesis is

derived. I was not involved in the production of this cell line nor have I been responsible for the production of any monoclonal antibody cell lines. However, the methods described below are those currently used by the University of Bristol, Department of Obstetrics and Gynaecology in the production of monoclonal antibodies to trophoblast antigens.

Preparation of trophoblast membrane extract for use in the immunisation of mice

This method was adapted from that originally described by Smith et al (1974) and Standing and Williams (1978).

A term placenta was obtained from the Central Delivery Suite within 30 minutes of delivery and the cotyledons cut into small pieces. The amnion, chorion, blood vessels and umbilical cord were discarded. The cut cotyledons were washed in 2L 0.1M  $\text{CaCl}_2$  and 1L P.B.S., shredded in a mincer and suspended in 350ml of 0.15M NaCl. This suspension was stirred for 30 minutes at 4°C to separate the chorionic villi from the bulk of the placental tissue. The mixture was sieved, the fluid collected and centrifuged at 3000 r.p.m. for 10 minutes and finally at 18,000 r.p.m. for 30 minutes. The pellet containing trophoblast membrane was saved and resuspended in 100ml per 500gm wet weight of placenta of 2% Tween 40 in 0.15M NaCl, 0.01M Tris/HCl pH 7.4, homogenised and stirred for 1 hour at 4°C. 20ml of 32% sucrose in 0.01M Tris/HCl pH 8.0 were loaded under 15ml aliquots of the preparation and centrifuged at 18,000 r.p.m. for 2 hours to remove serum proteins. The membranous band was removed from the surface of the sucrose, washed in 0.01M Tris/HCl (pH 8.0) and stored at -20°C until used.

### Mouse Immunisation Schedule

4-5 week old B alb/c mice were immunised by the subcutaneous injection of 50ug of trophoblast membrane preparation, in complete Freund's adjuvant, into the abdominal wall on two occasions four weeks apart. Two weeks after the second injection a further 50ug of trophoblast membrane preparation in 100ul of P.B.S. was injected into the tail vein and the mouse sacrificed three days later.

### Fusion of mouse spleen cells with N.S.I. mouse myeloma cells

#### a) Reagents

- 1) RPM I 1640 (Flow) is a cell culture medium which is supplied with or without a 20mmol Hepes buffer.
- 2) Fetal calf serum (Gibco) (F.C.S.) is heat inactivated and virus free. It is used in RPM I 1640 at a 10% dilution.
- 3) Glu/P/S is a 200mM solution of L-glutamine (Flow) in 100mls, to which 1,000,000 units of Benzyl Penicillin (Glaxo) and 1gm of Streptomycin (Glaxo) have been added. 5ml of glu/P/S is added to every 500ml RPM I 1640 used.
- 4) H.M.T. supplement consists of 20ml of a 0.01M hypoxanthine, 1.6ml of 0.001M methotrexate, 3.2ml of 0.01M thymidine and 0.6ml of 0.01M glycine. 1 x H.M.T. is 1.25ml of H.M.T. added to 100ml of cell culture medium. It is sterilised by filtration.

#### b) Method

The fusion was performed in an unidirectional laminar flow microbiological safety cabinet.

The spleen was removed from the dead immunised mouse and fragmented to form a single cell suspension in 5ml RPM I 1640 Hepes + F.C.S. + glu/P/S. This suspension was added to 5ml RPM I 1640 Hepes + F.C.S.. In order to estimate the number of cells in this suspension 100ul was made into a 1 in 50 dilution with 0.85%  $\text{NH}_4\text{Cl}$  to lyse any red blood cells. 500ul of this solution was mixed with an equal volume of 2% Trypan Blue in P.B.S. to stain any dead cells and an aliquot transferred to a counting chamber. There were usually between 2 and  $2.5 \times 10^8$  cells in the original solution. The initial suspension was centrifuged at 1000 r.p.m. for 5 minutes, the supernatant discarded and the cells resuspended in 5ml RPM I 1640 Hepes + F.C.S..

N.S.I. myeloma cells were grown up in RPM I 1640 Hepes + F.C.S. + glu/P/S for 24-48 hours and a volume containing 10% the number of spleen cells was centrifuged at 1000 r.p.m. for 5 minutes and resuspended in 5ml RPM I 1640 Hepes + F.C.S..

The two suspensions were mixed for 5 minutes at  $37^\circ\text{C}$ , centrifuged at 1000 r.p.m. for 5 minutes, the supernatant was discarded and 800ul of sterile 50% polyethyleneglycol in RPM I 1640 Hepes added over 1 minute. The solution was stirred at  $37^\circ\text{C}$  and fusion between spleen cells at the lymphoblast stage and N.S.I. cells occurs. 10ml of RPM I 1640 Hepes was added dropwise over 5 minutes to dilute the polyethyleneglycol and stop it clumping. The solution was centrifuged at 1000 r.p.m. for 5 minutes and the cells suspended in 4ml of RPM I 1640 Hepes + F.C.S. + glu/P/S + 1 x H.M.T., which was divided equally among 8 universal containers of 8ml RPM I 1640 Hepes + F.C.S. + glu/P/S + 1 x H.M.T.. 250ul of these solutions were added to each well of 8 96 well plates (Flow), which were sealed and incubated in a moist atmosphere containing 5%  $\text{CO}_2$  at  $37^\circ\text{C}$  for 10-12 days.

### Cloning

10-12 days after fusion culture supernatants were tested immunohistologically for antibody binding to trophoblast membrane. Positive supernatants were checked against normal human liver and kidney. Cultures secreting antibody not absorbed by these tissues were diluted using RPM I 1640 H.T. medium into microtitre plates at 10-20 cells/well with  $2.5 \times 10^5$  mouse thymocyte feeder cells. After growth and reassay cells were rediluted in RPM I 1640 into microtitre plates at 1-5 cells per well with thymocyte feeders. The process was repeated until wells containing single active clones were obtained. The cells were pooled, recloned and grown up as a single cell line. In order to ensure successful cloning cells were diluted into microtitre plates to give an average of one to two clones per well and supernatants checked for antibody to trophoblast membrane.

### 2.3 The NDOG2 monoclonal antibody

NDOG2 is a class IgG2b antibody and was produced using a technique similar to that described in 2.1.3 (Sunderland et al 1981). Indirect evidence that NDOG2 recognises a P.L.A.P. determinant comes from the immunohistological examination of human placentae. Positive reactive staining with NDOG2 first appears on placentae of gestational age 10-16 weeks and is present on all placentae of greater than 16 weeks gestation. Positive reactive staining was recorded on all of 50 term placentae examined and this distribution is identical to that of P.L.A.P.. The immunohistological technique employed and the distribution of reactive staining with NDOG2 in both malignant and non malignant tissues is described in detail in Chapter 3. Bowel, liver and kidney do not show any reactive staining with this antibody, evidence that NDOG2 does not cross react with either the intestinal or tissue non-specific isoenzymes.

Antibody specificity was demonstrated using serial dilutions of NDOG2 and 3 control antibodies whose properties are described in Table 3.1, in a solid phase enzyme immunoassay (McLaughlin et al 1983) which is described in detail in Chapter 4. Multiwell plates were coated with rabbit anti-mouse immunoglobulin, washed and incubated with serial dilutions of the appropriate monoclonal antibody. The wells were washed and incubated with 50ug/well of purified P.L.A.P., supplied by Dr R Sutcliffe (University of Glasgow). After a final wash a colourmetric reaction for alkaline phosphatase was demonstrated using paranitrophenol phosphate; only the wells containing NDOG2 showed any evidence of alkaline phosphatase activity (fig 2.2). Similar experiments using pregnancy serum in the second incubation again showed NDOG2 to specifically find a heat stable phosphatase activity.

Dr J L Millan of the Umea University, Stockholm supplied independant confirmation that NDOG2 only recognises the placental isoenzyme of alkaline phosphatase and that it recognises the 3 common allelic forms of P.L.A.P. equally well.

## 2.4 Potential uses of monoclonal antibodies

### 2.4.1 Immunohistology

#### Methods available

Immunohistological examination of tissues can be performed using monoclonal or polyclonal antibodies. The antigen binding region of a monoclonal antibody lacks the variability present in polyclonal antibodies and is therefore highly specific. A further advantage of monoclonal antibodies over polyclonal antisera is that limitless supplies of exactly the same antibody can be easily produced (Mason et al 1982).



There are three main methods of immunohistology (Kurman 1984).

1) Indirect immunoperoxidase technique

The sections are incubated with the primary antibody (e.g. of mouse origin) and then with a peroxidase conjugated immunoglobulin directed against the primary antibody (e.g. a rabbit anti mouse immunoglobulin). The reaction product is visualised by the addition of diaminobenzide (D.A.B.) and hydrogen peroxide ( $H_2O_2$ ).

2) Peroxidase anti-peroxidase (P.A.P.)

This method depends on an excess of rabbit anti mouse immunoglobulin which links the primary mouse antibody to the mouse antibody in the P.A.P. complex, which is a soluble antigen-antibody complex composed of peroxidase and a mouse antibody to peroxidase. The reaction is visualised by the addition of D.A.B. and  $H_2O_2$ . This method has increased sensitivity over the indirect peroxidase method but mouse P.A.P. is not widely available from commercial sources due to the difficulty in producing large volumes of polyclonal antibody in mice (Mason et al 1982).

3) Biotin-Avidin method (Hsu et al 1981)

Avidin, an egg white glycoprotein, has a very high affinity for the vitamin biotin. Biotin is co-valently bound to peroxidase molecules allowing peroxidase to bind avidin. The complex is linked to the primary antibody by a biotinylated antiprimary immunoglobulin and the reaction product visualised by the addition of D.A.B. and  $H_2O_2$ . The biotin-avidin method is the most sensitive of the three methods and reduces the risk of non-specific staining.

#### 4) Immunofluorescence

Immunofluorescence can be used as an alternative to immunoenzymatic methods of demonstrating antigen/antibody reaction. It is easy to use but does not allow simultaneous visualisation of the morphology of the section. In addition fluorescently labelled sections tend to fade on storage and non-specific background staining is common especially if the antibodies are over conjugated (Mason et al 1982).

#### Limitations of Immunohistological labelling (Mason et al 1982)

##### 1) Limited sensitivity

If the sought antigen is only produced in limited amounts or has a very short half life then it may not be detectable. Also if the antigen has been denatured by the fixative (particularly Zenker-type fixatives and to a lesser extent 10% formalin and Bouins) then a false negative reaction may be obtained. This can be a particular problem with paraffin embedded tissues and so cryostat sections are frequently used, but there is still a risk of destruction of the antigen when the sections are fixed prior to staining. Acetone has been shown to be the best fixative in these circumstances (Gomori 1952, Dempo et al 1980).

##### 2) Background staining

There is much less of a problem with monoclonal as opposed to polyclonal antibodies, but nonetheless still occurs. It can be recognised by the use of appropriate controls and thereby differentiated from the presence of cellular antigen in the extracellular environment, e.g. in gland secretions.



The Histochemical method of detecting Alkaline Phosphatase  
 Histochemical techniques of tissue staining can be applied in the case of alkaline phosphatase. This method depends on the substrate alphanaphthyl acid phosphate being split by alkaline phosphatase and the product being visualised by fast blue BBN (Sasaki and Fishman 1973) or fast red violet L.B. salt (Nozawa et al 1980) as opposed to an antibody recognising a specific determinant. This method requires the presence of the enzyme in an active form and therefore appropriate aminoacid inhibitors are usually used to differentiate between the various isoenzymes.

#### 2.4.2 Serum Assay

Enzyme linked immunosorbant assays (E.L.I.S.A.) and radio-immunoassays (R.I.A.) are the two methods in common usage employing antibodies in serum assays. Both depend on the adsorption of either antigen or antibody to a solid phase, which is a result of hydrophobic interactions between non polar protein substances and the non polar plastic matrix (Clark and Engval 1980). The solid phase may be particles of cellulose or polyacrylamide but, as these require centrifuging in the washing steps, preformed material such as beads, tubes, discs or microplates are more conveniently used. Polystyrene is the most commonly used solid phase as it can be coated easily and reproducibly.

#### E.L.I.S.A.

A variety of assays exist (Voller et al 1976).

##### A) Competitive method

i) Specific antibody is attached to the solid phase which is then washed.

ii) A test solution thought to contain antigen is then added. It is either mixed with enzyme-labelled antigen or enzyme-labelled antigen is added after a short time. Plates are then incubated and then washed.

iii) Enzyme substrate is added. The colour change is inversely proportional to the amount of antigen present in the test sample.

B) Double Antibody Sandwich Method

i) As (A).

ii) The test solution is incubated with the sensitised solid phase and then washed.

iii) Enzyme-labelled specific antibody to the antigen is then incubated with the solid phase and then washed.

iv) Enzyme substrate is added. The colour change is proportional to the amount of antigen in the test solution.

C) Modified Double Antibody Sandwich Method

i) As (B).

ii) As (B).

iii) Specific antibody, produced from a different species than the antibody in step (i) is added, incubated and washed.

iv) Enzyme labelled anti immunoglobulin is then added, incubated and washed. This anti immunoglobulin is reactive to the antibody in step (iii) but not to that in step (i).

v) Enzyme substrate is added and the colour change is proportional to the amount of antigen in the test sample.

D) Inhibition E.L.I.S.A.

i) The relevant antigen is attached to the solid phase and then washed.

ii) The test solution is mixed with a reference antiserum containing specific antibody and this mixture is incubated with the sensitised solid phase, which is then washed. If this antibody is enzyme labelled then step (iii) is not required.

iii) Enzyme labelled anti immunoglobulin (reactive with antibody in (ii) is added, incubated and washed.

iv) Enzyme substrate is added. The amount of antigen present in the test sample is indirectly proportional to the colour change.

In these assays the product of the enzyme causes a colour change which can be measured using a spectrophotometer. Two enzymes are commonly used, alkaline phosphatase and peroxidase. The substrate for alkaline phosphatase is paranitrophenyl phosphate, the reaction is stopped with concentrated NaOH and the resultant stable yellow product

measured photometrically at 405nm. The best substrate for peroxidase is O-phenylenediamine (O.P.D.), the reaction can be stopped with 2M  $\text{H}_2\text{SO}_4$  and read at 492nm. O.P.D. has been reported as being mutagenic and should be used with care.

Where alkaline phosphatase is the antigen to be measured in serum then enzyme labelled antibodies are not necessary to develop a colour reaction (McLaughlin et al 1983).

R.I.A. makes use of the radionuclide Iodine-125 ( $^{125}\text{I}$ ) in place of enzyme labelled antibodies/antigens, but otherwise the various methods are similar.

There are a number of problems associated with E.L.I.S.A. (Clark and Engvall 1980):

- 1) As the antibody (or antigen) is only physically adsorbed to the solid phase and not co-valently bound then the protein can be lost in washes and incubation thereby lowering the precision and sensitivity of the assay, particularly in competitive assays.
- 2) The adsorbed protein may become denatured thereby losing some binding capacity.
- 3) The binding of protein to the solid phase is non-specific and therefore either the test antigen, in competitive and sandwich assays, or the second antibody can also bind. This can be minimised by blocking the remaining sites on the solid phase after step (1) with bovine serum albumin (B.S.A.) or F.C.S..

### 2.4.3 Radioimmunoscintigraphy

#### Historical background

Paul Ehrlich, in the early part of this century, first proposed the idea of using antitumour antibodies to selectively target toxic substances to tumour cells (Himmelwiet 1960). However, it was not until 1929 that tumours were shown to be antigenically distinct from non malignant tumours (Witebsky 1929), and their ability to take up foreign proteins and dyes non-specifically was demonstrated by Duran-Reynals (1939). Pressman and Keighley (1948) used a radiolabelled antibody in animal experiments to visualise the kidney. Bale et al (1957) and Pressman (1957) demonstrated radiolabelled antibody uptake by transplanted rodent tumours and tumour antibodies were shown to cross react with normal tissues by Day (1964). In the early 1970's human tumours transplanted in hamsters were visualised using radiolabelled anti H.C.G. (Quinones 1971) and anti C.E.A. (Goldenberg et al 1974, Primus et al 1973). Both these antibodies were only partly purified, by using affinity purified antibodies, again in animal experiments, Mach (1974) obtained superior tumour visualisation. All these experiments demonstrated a raised concentration of anti-tumour antibody, when compared with a non-specific antibody in the relevant tumour.

### Radioimmunodetection of tumour deposits

A large number of human cancers have been investigated using radioimmunoscintigraphy (R.I.S.), these include breast (Rainsbury et al 1983, Epenetos et al 1982), colon (Goldenberg et al 1980, Farrands et al 1982, Epenetos et al 1982, Chatal et al 1984), islet cell tumour of the pancreas (Fairweather et al 1982), melanoma (Larson et al 1984), ovary (Goldenberg 1980, Van Nagell et al 1980, Halsall et al 1981, Epenetos et al 1982 and 1984), osteosarcoma (Perkins et al 1984), prostate (Deland and Goldenberg 1984, Zimmer et al 1982), testis - germ cell tumours (Halsall et al 1981) and thyroid (Shepherd et al 1984). R.I.S. is usually performed after intravenous injection of the labelled antibody but intralymphatic injection has also proved to be useful and this will be discussed separately. Intracavity injection of radio-labelled antibodies has been used therapeutically but not diagnostically (Hammersmith Oncology Group 1984).

### Choice of Antibody

Antibodies have been raised to a variety of tumour antigens, some of these such as C.E.A. (Goldenberg et al 1978) and human milk fat globule membrane antigen (Epenetos et al 1982) are present on normal human tissues and could lead to production of false positive results. Other antibodies raised against tumour tissue e.g. anti P 97 (Larson et al 1984) and 17-1 A (Chatal et al 1984) tend to be more tumour specific.

Among the types of antibody which have been used are:

- 1) Unpurified polyclonal antisera which were used in some early animal experiments but gave poor results and were consequently abandoned.

2) Affinity purified antibodies which have the disadvantage that several antigenic sites may be recognised and have therefore the potential for unpredictable cross reactions with other antigens.

3) Monoclonal antibodies. These, in theory, should be superior to (2) as their specificity and affinity are precisely determined. Initial reports suggested that this may be so (Ballou et al 1979, Moshakis et al 1981). However, since then a number of workers have not, in practice, found any superiority for monoclonal antibodies (Fairweather et al 1982, Begent 1983, Jeppsson et al 1984).

4) Fab and  $F(ab')_2$  fragments of antibodies are produced by papain or pepsin digestion of whole antibodies, and in consequence neither bear the antigenic Fc receptor and therefore do not bind with complement or cells bearing Fc receptors. They, therefore, do not form immune complexes as readily as whole antibodies and should consequently produce less background activity. In practice however, immune reactions still occur (Vinocur 1984) and while  $F(ab')_2$  fragments give optimal background ratios for earlier imaging, intact antibodies tend to give the best tumour images (Khow et al 1984). Nonetheless tumour deposits as small as 1.5cm have been successfully visualised using labelled  $F(ab')_2$  fragments (Romanelli-Beardsley et al 1983) and Fab fragments (Larson et al 1984).

### The Choice of Radionuclide

The ideal radionuclide for radioimmunoscintigraphy would:

- a) Bind easily to the appropriate antibody without reducing its specific activity;
- b) Not dissociate from the antibody when injected intravenously;
- c) Be rapidly excreted without accumulating in any organ when the radiopharmaceutical is broken down by the reticulo-endothelial system;
- d) Be a pure gamma emitter with an energy range suitable for the modern gamma camera;
- e) Have a sufficiently long half life to give the antibody time to reach and bind to the appropriate tumour antigen but not so long that it may present a hazard to the patient's health.

In practice the ideal radionuclide does not exist. However, several have been successfully used in radioimmunoscintigraphy, the most common of which are the three isotopes of iodine -  $^{131}\text{I}$  (Hoffer et al 1974, Goldenberg et al 1980),  $^{123}\text{I}$  (Epenetos et al 1982) and  $^{125}\text{I}$  (Schlumberger et al 1982). In addition 111-Indium ( $^{111}\text{In}$ ) (Fairweather et al 1983, Slack 1984) and 99m-Technecium ( $^{99\text{m}}\text{Tc}$ ) (Wong et al 1982) have been successfully used. The properties of these various isotopes are summarised in Table 2.1.



$^{123}\text{I}$  and  $^{111}\text{In}$  are the most suitable radionuclides in that they have the best energy ranges for the modern gamma camera and both are pure gamma emitters.  $^{131}\text{I}$  with its high energy range and combined beta and gamma emission gives poor quality images.  $^{99\text{m}}\text{Tc}$  and  $^{125}\text{I}$  have respectively too short a half life and too low an energy emission to be of use when labelled to antibodies in humans.

$^{123}\text{I}$  was the radionuclide used in this thesis as blocking agents, such as potassium iodide or potassium perchlorate can be used to reduce uptake of the free radionuclide by normal organs; no such blocking agents exist for  $^{111}\text{In}$ .  $^{123}\text{I}$  is cheaper than  $^{111}\text{In}$  but because of its short half life and limited production by Harwell, radioimaging is limited to one day of the week.

#### Comparison of R.I.S. with other imaging techniques

R.I.S. is likely to have its biggest impact in the detection of intra-abdominal tumour deposits. The two most commonly used imaging techniques in these cases are Xray computed tomography (C.T.) and ultrasound scanning (U.S.S.) both of these have relatively poor sensitivity for small solid metastases, particularly after primary surgery (Piver 1983).

R.I.S. is at least as good as conventional imaging techniques for abdominal tumours (Begent 1984) and gives more information than C.T. as to whether the deposits are viable or necrotic (Begent 1983). It may be as good as C.T. in cases of lung metastases (Begent 1983) although other workers (Halsall et al 1981) have found C.T. superior in these cases.

Several studies exist reporting R.I.S. detection of intra-abdominal deposits not detected by C.T. and U.S.S. (Van Nagell et al 1980); C.T., angiography and intravenous pyelography (Goldenberg et al 1980); C.T. and angiography alone (Fairweather et al 1982) or C.T. and lymphangiography (Halsall et al 1981).

While in animal experiments the smallest tumour deposit detected was only 1mm in diameter (Epenetos et al 1984) in humans this ranges from 1.5cm (Fairweather et al 1982, Larson et al 1983) to 2cm (Silva et al 1980, Van Nagell et al 1980, Halsall et al 1981).

#### Problems associated with R.I.S.

##### Allergic Reactions

There is always a risk of the development of allergic reactions when a foreign protein is injected. This would not appear to be a great problem. Sfakianakis and Deland (1982) only reported 2 reactions in over 400 examinations, in 50 of which repeat injections (up to 3 times) were given without side effects. In this case only small quantities 150-250ug of antibody were used. However, the development of human anti mouse antibody (H.A.M.A.) has been reported even with Fab fragments but tends to occur only when large amounts of antibody are used - up to 20mg in one series (Larson et al 1983). The presence of H.A.M.A. causes more rapid clearance of the labelled antibody with a subsequent lower uptake by the tumour and is most easily avoided by using low (less than 400ug) doses of antibody per study (Vinocur 1984). Nonetheless, it is sensible precaution to perform skin testing before the injection of repeat doses of antibody.

### Specificity

Tumours, because of the increased permeability of their capillaries, tend to absorb more foreign protein from the circulation than normal tissues (Duran-Reynals 1939). It is, therefore, important to demonstrate an antibodies specificity in vivo, this is usually performed by using  $^{99m}\text{Tc}$  labelled human serum albumin (Goldenberg et al 1978) or a labelled non-specific antibody (Van Nagell 1980). The uptake by the tumour of the radiolabelled antibody is however low, being reported between 0.1-7% of the injected dose (Mach et al 1980, Carroll et al 1984).

### High background activity

There is usually only a small percentage of the injected radiopharmaceutical that localises to the tumour and because of this there is a high background activity. This is particularly a problem in the liver where up to 40% of the whole body radioactivity is present 24 hours after the initial injection of  $^{111}\text{In}$  (Rainsbury et al 1983) and can lead to false positives in this organ (Begent 1983).

Iodine can be absorbed by an incompletely blocked thyroid gland, gastric mucosa and by scars (Goldenberg et al 1980, Mach et al 1980).

A variety of antibodies raised to highly tumour restricted antigens exist (Larson et al 1983, Farrands et al 1982). However others e.g. human milk fat globule membrane antigen are present, in smaller quantities, on normal secretory epithelium as well (Epenetos et al 1982) and can therefore contribute to high background activity. Immune complexes between circulating antibody and antigen e.g. H.C.G.,

(Goldenberg et al 1980) or C.E.A. (Mach et al 1980) might be expected to collect in the liver and give rise to false positives. This, in fact, happens infrequently and when it does gives rise to a picture of diffuse "involvement" but not discrete nodules. No case of serum sickness has been reported secondary to these complexes.

A number of different methods have been suggested to enhance the visualisation of areas of increased activity, although these are often identifiable, even in liver, without the need for enhancement (Romanelli-Beardsley 1983).

The first method of enhancement was that of subtraction using  $^{99m}\text{Tc}$  labelled human serum albumin to give the blood pool picture. By subtracting this image from that obtained with the labelled antibody a clearer picture of areas of increased activity was obtained (Goldenberg et al 1978). This is not an ideal method as the  $^{131}\text{I}$  image is larger than that of  $^{99m}\text{Tc}$  and does not have the same uniformity of response (Begent 1983) and a number of artefacts have been generated using this method (Green et al 1984). A better method of subtraction involves subtracting the early (post injection) image, giving the blood pool picture, from a later image which will represent tumour uptake of the radiopharmaceutical as well as blood pool. However, accurate repositioning of the patient for each image is essential to avoid the generation of artefacts (Carroll et al 1984).

Liposomally entrapped second antibodies (L.E.S.A.) have been used to increase the clearance of the first antibody from the blood pool without affecting clearance from the tumour (Begent et al 1982). The second antibody is raised against the first antibody species type and does give improved images, however, as L.E.S.A. is cleared by the reticulo-endothelial system high liver background images may be generated. Antibody fragments, as discussed earlier, are cleared from the circulation more rapidly than whole antibody and give less background activity.

Better imaging techniques such as singlephoton emission tomography, may in the future lead to improved images (Perkins et al 1984).

#### False negative results

These have been variously reported as between 10% (Goldenberg et al 1980) and 60% (Mach et al 1980). One of the main limiting factors seems to be the size of the tumour deposit, the smallest metastasis detectable being between 1.5cm (Vinocur 1984) and 2cm (Silva et al 1980, Van Nagell et al 1980).

In the case of boney metastases R.I.S. seems to be best in detecting early deposits (Rainsbury et al 1983) older, larger metastases detected by Xray or conventional bone scans are not visualised (Rainsbury et al 1983, Deland and Goldenberg 1984).

The blood brain barrier results in the poor visualisation of cerebral metastases but nonetheless it is possible to detect some intracranial tumour deposits by R.I.S. (Farrands et al 1982, Richardson et al 1984).

#### Radioimmunoscintigraphy in Ovarian Tumours

In both germ cell and epithelial tumours of the ovary anti alphafoetoprotein has been successfully used in the detection of metastases (Kim et al 1980, Halsall et al 1981). Other antibodies used for R.I.S. in epithelial ovarian cancers include anti C.E.A. (Goldenberg et al 1980), an affinity purified antibody to C.E.A., which successfully detected all the primary tumour sites but only 67% of the metastatic sites by R.I.S.. Nodules of less than 1cm diameter were the main cause of these false negatives. Many of these patients had high serum C.E.A. levels but this did not lessen the sensitivity of the investigation. Similar findings were reported by Van Nagell et al (1980) who also successfully imaged 1 from 5 benign ovarian cysts; again plasma levels of C.E.A. were not an indicator of how successful the procedure would be.

A monoclonal antibody to human milk fat globule membrane antigen designated H.M.F.G.<sub>2</sub> labelled with <sup>123</sup>I, has been used in R.I.S. of ovarian cancers. This antigen is found on all normal human secretory epithelial cells but is present in much larger quantities in various malignant tumours including ovary (Epenetos et al 1982). In this study over 70% of tumour deposits were detected by radioimmunoscintigraphy, those that were not visualised included abdominal, chest and lumbar spine tumours. Tumour

uptake was between 0.2 and 2.6% of the injected dose with radiolocalisation occurring between 10 minutes and 18 hours from the time of injection (Epenetos et al 1984). Using the same antibody in a prospective preoperative trial good correlation between R.I.S. and operative findings was obtained in 17 from 18 cases but uptake of the labelled antibody was also reported in benign ovarian cysts and uterine fibroids (Granowska et al 1984).

#### R.I.S. using antibodies to P.L.A.P.

Apart from NDOG2 a variety of monoclonal antibodies to P.L.A.P. are being used in the radioimmunodetection of ovarian cancer deposits e.g. the H317 and H6/72 monoclonal antibodies (Advances in the application of monoclonal antibodies in Clinical Oncology, Royal Postgraduate Medical School 1985). However, no substantive series has yet been published. Part of this thesis explores the use of radiolabelled NDOG2 in the radiolocalisation of ovarian cancer deposits.

#### Lymphography using Radiolabelled Antibodies

This was first used to locate nodes involved with Hodgkins Lymphoma (Order et al 1975). Involved nodes were demonstrated but the possibility of false positive results due to the concentration of a foreign antibody by the lymphatics, was raised. Deland et al (1979) used intralymphatic injection of  $^{131}\text{I}$  anti C.E.A. to detect axillary node involvement by breast cancer, however, a high false positive rate was recorded which the authors suggested was due to the presence of C.E.A. in nodes draining the tumour site. But this could equally well have been due to non-specific



uptake by the lymph nodes. Begent (1984) suggested that blockage of lymphatics by tumour could limit the usefulness of this procedure.

However, one advantage of radioimmuno-lymphography has over intravenous injection of labelled antibody is the minimal binding of antibody to antigen in the circulation or on normal tissue. Weinstein et al (1983) in animal experiments demonstrated a fourfold increase in uptake of labelled antibody by lymph node metastases when an intralymphatic route was used in preference to intravenous injection. This suggests that the low uptake of labelled antibody by tumour previously recorded is not due to saturation of antigenic sites but to low concentrations of available antibody in the circulation (Begent 1984). Large intravenous doses of antibody have been used (up to 20mg) in R.I.S. of colonic and ovarian cancer but these have not improved the quality of the images (Larson personal communication).

#### 2.4.4 Immunotherapy

This can take the form of a non-specific boost to the patient's immune system or as antibody directed therapy to the tumour.

##### 1) Non-specific immuotherapy

The rationale for this form of therapy is to reverse the immune depression that can exist with many tumours or that results from conventional therapy - surgery, chemotherapy



or radiotherapy; in addition it may be possible to increase the immunogenicity of the tumour cells themselves (Freedman et al 1983). The agents used include Bacille Calmette-Guerin (B.C.G.) (Hudson et al 1976), autologous tumour extracts and corynebacterium parvum (Gudson et al 1983), and virus modified tumour cell extracts (Freedman et al 1983). The benefit of this form of therapy is debatable (Hamblin 1982).

## 2) Antibody directed therapy

Chlorambucil, linked to antibody, was an early form of antibody directed therapy. This was not successful as the complex was not stable when given to the patient (Rubens and Dulbecco 1974).

Toxin conjugated antibodies would appear to be an ideal chemotherapeutic agent. Toxins postulated for use include ricin (Krolik et al 1982) or diphtheria toxin (Moolten et al 1975). Ricin, in particular, appears an attractive toxin to use in vivo. It consists of a toxic polypeptide ('A' chain) coupled to a cell binding polypeptide ('B' chain) (Olsnes and Phil 1973). The 'A' chain by action on the ribosome, kills the cell by inhibiting protein synthesis (Olsnes and Phil 1984) but 'A' chain on its own cannot enter the cell and is therefore harmless (Neville and Youle 1982). 'A' chain ricin has been successfully linked to antibodies (Uhr and Vitella 1983, Seon et al 1983) and has been used to eliminate leukaemia cells in animals (Uhr and Vitella 1983) and from marrow in man, prior to autologous transplantation (Seon et al 1983). Similarly, antibodies linked to magnetite containing polystyrene microspheres have been used to

eliminate neuroblastoma cells from marrow, by passing the suspension through a magnetic field, prior to autologous transplantation (Treleaven et al 1984) and complement fixing monoclonal antibody used to "cleanse" bone marrow, in vitro, of breast cancer cells (Buckman et al 1982).

While toxin conjugated antibody therapy may have a place in the treatment of leukaemias, therapy of solid tumours, such as ovarian carcinoma, present a number of problems:-

- i) Penetration of the tumour mass may not be adequate as many solid tumours are relatively avascular at their centres.
- ii) Ensuring that the antibody used recognises tumour cells alone. If the antigen is also expressed by normal tissues then the specificity of antibody directed therapy is lost. Unfortunately, no truly tumour specific antigen exists in the case of ovarian cancer, although some e.g. C.A. 125 (Bast et al 1981) are only weakly expressed by certain normal tissues (Kabawat et al 1983, Niloff et al 1984).
- iii) Expression of the antigen within the tumour is not always homogenous (Hammersmith Oncology Group 1984). Thus even if the cells expressing that particular antigen are destroyed a population of tumour cells will be unaffected, multiply and ultimately kill the patient.

iv) It has become apparent from experience obtained with R.I.S. that only a small percentage of the injected antibody will localise to the tumour (Epenetos et al 1982, 1984; Carroll et al 1984). As this is unlikely to be due to saturation of the available antigenic sites (Begent 1984) a higher dose of antibody could be given. However, this would increase the risk of an allergic reaction (Vinocur 1984) and lead to the development of antibodies which would tend to render this form of therapy less effective (Larson et al 1983, Vinocur 1984).

v) Antigenic modulation; cells exposed to an antibody can undergo change (modulation) so that they no longer express that antigen. This would not be a problem if every cell that comes into contact with a toxin conjugated antibody is destroyed, however a better form of therapy might involve the use of antibody fragments which appear not to induce modulation (Glennie and Stevenson 1982).

#### Antibody directed Radiotherapy

The treatment of patients with ovarian cancer by means of radioactive colloids is not new. Intra-abdominal instillation of  $^{32}\text{P}$  has proven to be as effective as other forms of therapy and has few side effects (Penzer et al 1978). However, homogenous distribution throughout the peritoneal cavity cannot be guaranteed, particularly if adhesions are present. The retroperitoneal tissues such as the para-aortic nodes receive no treatment and these can be involved even when no other extra ovarian spread is apparent (Piver et al 1978). Therapy with radionuclide linked antibodies would help to overcome the lack of homogeneity of expression of the antigen, by the tumour, as any cell within a few

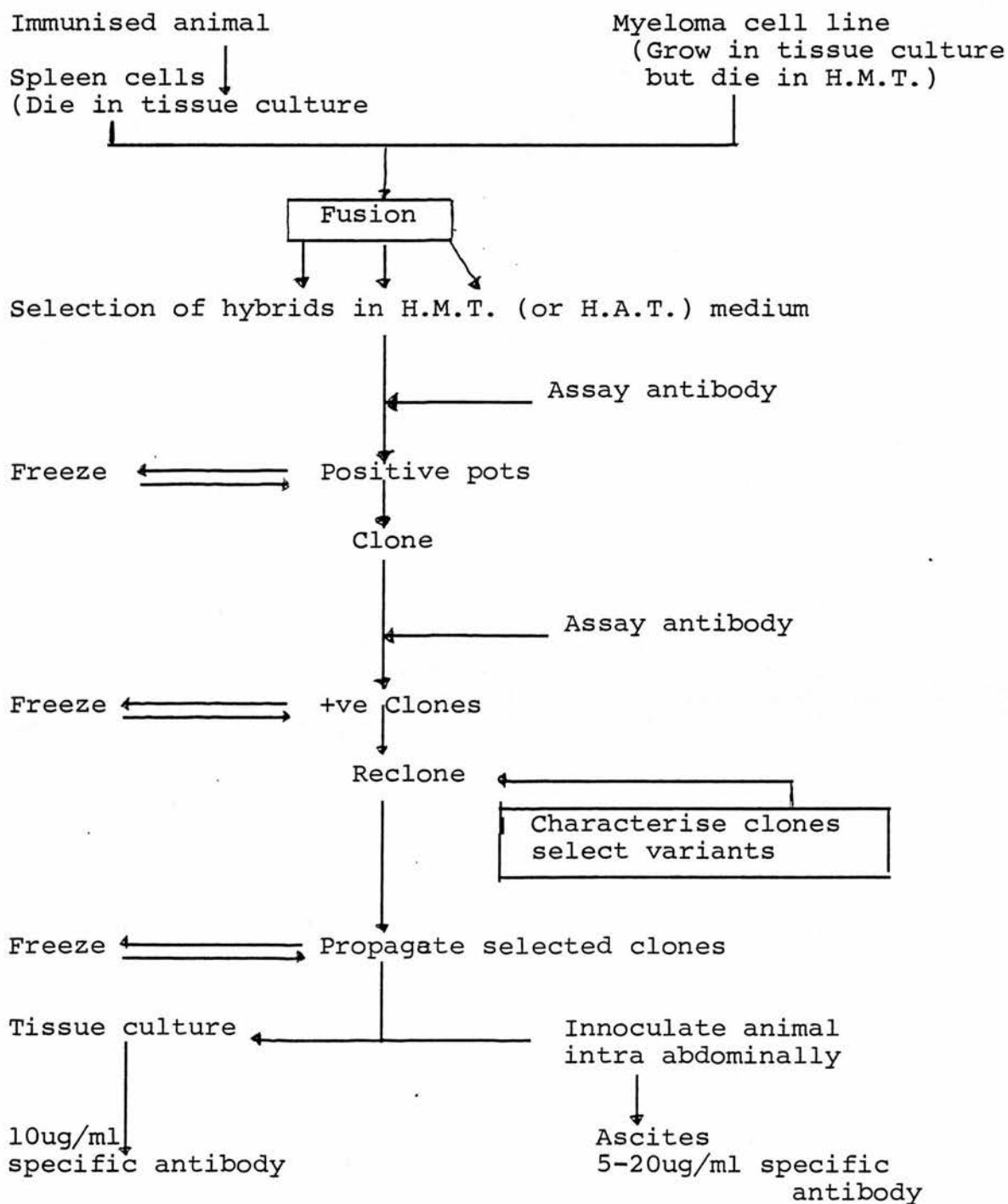
millimetres of a cell, with beta particle emitting radionuclide attached, would be destroyed. In addition administration of the antibody into a restricted space such as a pleural, pericardial or peritoneal cavity results in a much higher uptake of the antibody, by the tumour than would be anticipated by an intravenous injection (Hammersmith Oncology Group 1984).

The most obvious radionuclide to use in this situation is  $^{131}\text{I}$ , predominantly a beta emitter, which can be easily linked to proteins. There is a risk of loss of activity by the antibody when iodinated and from radioactivity of the radionuclide. Different monoclonal antibodies have been successfully iodinated with high doses of  $^{131}\text{I}$  by both the chloramine T (Ferens et al 1984) and iodogen methods (Hammersmith Oncology Group 1984) without significant loss of activity.

Three patients, one with an advanced ovarian carcinoma have been treated by intracavity instillation of  $^{131}\text{I}$  H.M.F.G.<sub>2</sub> antibody and remissions induced (Hammersmith Oncology Group 1984).

## 2.5 Tables, Charts and Figures

Fig 2.1 Usual steps involved in the derivation and cloning of antibody producing hybrid cells



From Galfe and Milstein (1981)

Table 2.1 Properties of the various radionuclides used in Radioimmunoscintigraphy

	$^{131}\text{I}$	$^{125}\text{I}$	$^{123}\text{I}$	$^{111}\text{In}$	$^{99\text{m}}\text{Tc}$
Half life	8 days	60 days	13 hours	3 days	6 hours
Particle emitted	Gamma and Beta	Gamma	Gamma	Gamma	Gamma
Energy range (kev)	364	35	160	180	140
Organs that accumulate free radionuclide	Gut mucosa Thyroid	Gut mucosa Thyroid	Gut mucosa Thyroid	<sup>4,5</sup> Bone Liver	Gut mucosa
Ease of binding to proteins	Easy	Easy	Easy	Easy	Easy
Effect of method of binding and radionuclide on the specific activity of the antibody	Up to <sup>3</sup> 40% loss of activity	Chloramine T <sup>1</sup> or Iodogen Chaelation			
			No loss <sup>2</sup> of specific activity	No loss of activity	
Ease of obtainability	Daily	Daily	Weekly	Weekly	Daily
Expense	+	+	+++	++++	+

1 = Goldenberg et al (1980)

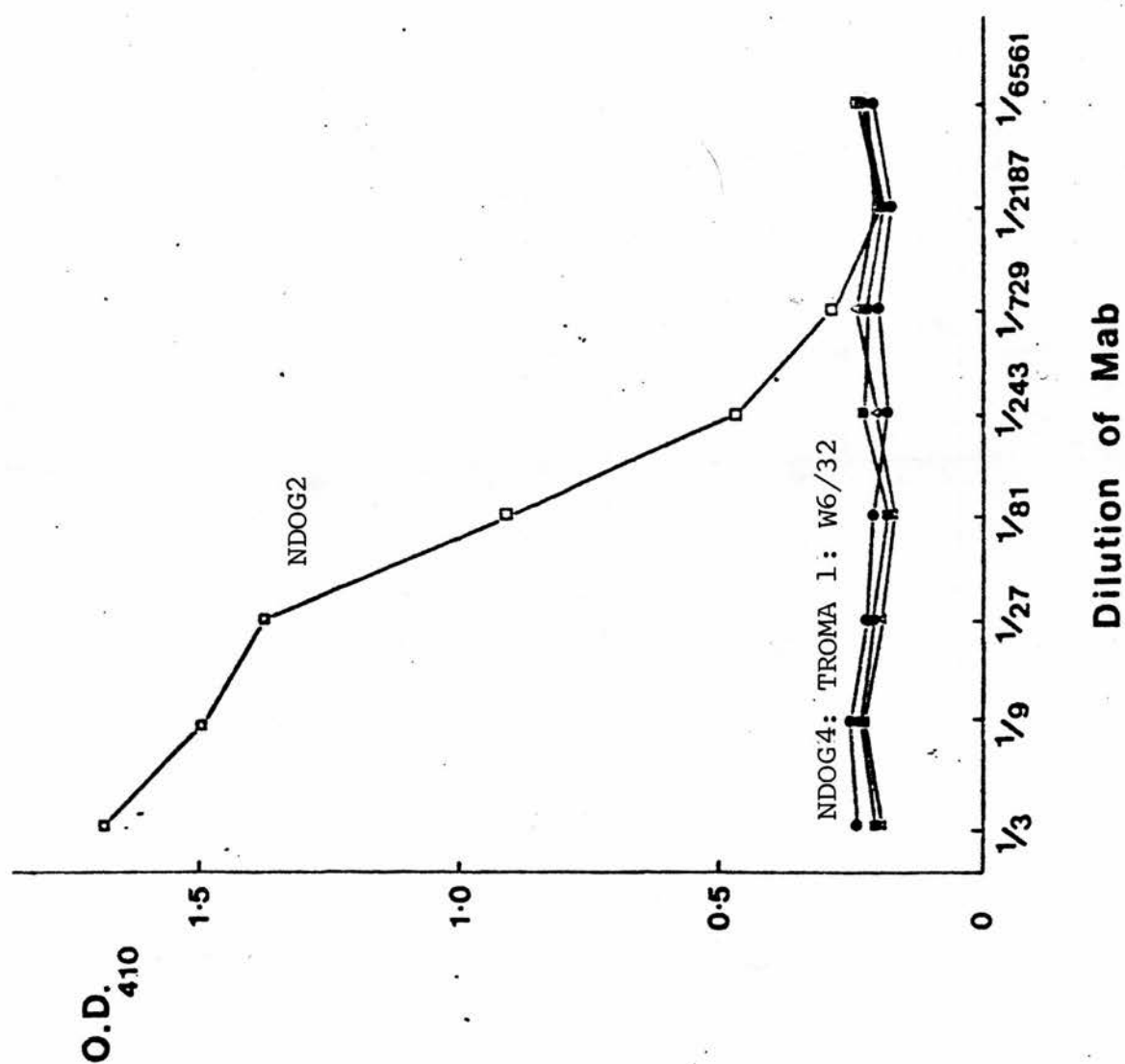
4 = Vinocur (1984)

2 = Epenetos et al (1982)

5 = Epenetos et al (1984)

3 = Larson et al (1984)

Fig 2.2 Demonstration of NDOG2 specificity



### CHAPTER 3

IMMUNOHISTOLOGY OF NORMAL TISSUES AND

GYNAECOLOGICAL MALIGNANCIES



### 3.1 Introduction

Immunohistology was performed, using an indirect immunoperoxidase technique on frozen sections of normal and malignant tissues in order to assess the distribution of the NDOG2 determinant in healthy organs and in gynaecological cancer. Histochemistry, using specific aminoacid inhibitors of the various isoenzymes of alkaline phosphatase was performed in certain tissues, to confirm that NDOG2 was recognising P.L.A.P..

Frozen sections were used in preference to formalin fixed, wax embedded tissue as it has been the Department's previous experience that conventional methods of fixation result in the partial denaturation of the NDOG2 determinant (Sunderland et al 1984).

### 3.2 Methods

#### 3.2.1. Tissue collection and storage

Tissue from normal gynaecological organs and tumours was obtained fresh at the time of operation. Normal endometrium was sampled by dilation and curettage from patients undergoing laparoscopy for sterilisation or investigation of subfertility. Postmenopausal endometrium was obtained from patients undergoing diagnostic curettage for postmenopausal bleeding in which subsequent histological examination showed no evidence of malignancy. Specimens of cervix, myometrium and fallopian tube were obtained from hysterectomy specimens, where no underlying malignancy was present.

Placental tissue was obtained, fresh, either at delivery or from therapeutic termination of pregnancy depending on the gestational age of the pregnancy.

Small pieces of tissue of 5mm diameter were snap frozen on aluminium foil floating in liquid nitrogen, within 2 hours of delivery or operation and stored, in cyrotubes, at  $-70^{\circ}\text{C}$  or in liquid nitrogen until used.

Other specimens of normal tissue were obtained from post mortem examination material, within 8 hours of death and similarly stored.

### 3.2.2 Immunohistology

Frozen sections of 6 $\mu$  thickness were cut and mounted on four well, teflon coated slides, air dried for 1 hour and fixed in acetone for 10 minutes at room temperature. One section from each block was fixed in 10% formol saline, stained with Mayers haematoxylin (Gurr) and 1% eosin (Gurr) and mounted in D.P.X. (B.D.H.). This section acted as a reference.

The other sections were washed in 0.15M NaCl, 0.05M Tris HCl pH 7.4 (T.B.S.) for 2 minutes. 50ul of NDOG2 (2 wells per slide) or an appropriate positive or negative control (1 well per slide) (Table 3.1) were applied to each section and incubated for 45 minutes at room temperature in a moist atmosphere. They were then washed twice, for 2 minutes in T.B.S. and similarly incubated with 50ul of peroxidase conjugated rabbit anti mouse immunoglobulin (Dako) at 1/50 dilution in T.B.S. with 10% normal human serum for 30 minutes. A further washing was performed and the sections incubated, in a fume cupboard, with 50ul of diaminobenzide tetra HCl (Sigma) at 0.5mg/ml in T.B.S., with 0.0075%  $\text{H}_2\text{O}_2$  (Analar) for 8 minutes. They were then washed in excess water, counterstained with Mayers haematoxylin and mounted in D.P.X..

A positive control antibody was used to indicate the antigenic integrity of the tissues and the technical validity of the experiment. Antibodies which were used were W6/32, BMH 1 and TROMA 1. The negative control antibodies, NDOG4, BMH5 and MRC OX6 were used to identify any non-specific binding of antibodies (see Table 3.1 for reactions and sources of these antibodies). This was negligible except for cervical columnar epithelial secretions and to a lesser extent colonic epithelium, which could be eliminated by the inclusion of normal human serum (10-50%) in the first incubation buffer. This had no effect on the specificity of NDOG2 as assessed on placental sections. A small population of cells with endogenous peroxidase activity were identified in the thymus and in the lamina propria of duodenum and colon. The morphology position and staining intensity of these cells obviously distinguished them from specifically staining tissue. Endogenous peroxidase activity was also noted in erythrocytes.

### 3.2.3 Histochemistry

Frozen sections of 6µ thickness were cut and air dried for 1 hour at room temperature. No fixative was applied. Napthyl A.S. M.X. phosphoric acid (2mgm) (Sigma) was dissolved in 0.2ml dimethylformamide and 9.8ml of 0.1M Tris HCl pH 8.0 added. 10mgm of Fast Blue (Gurr) was added, the sample divided into 1ml aliquots and 1ml of the appropriate inhibitor added to each, centrifuged at 12,000 r.p.m. in a Beckman microfuge for 2 minutes and applied to the tissue sections. The sections were incubated for 15 minutes at room temperature, washed in excess tap water and mounted in glycerin jelly.

1mM Levamisole was used to inhibit tissue non-specific alkaline phosphatase and 2mM L-phenylalanyl glycylglycine used to inhibit P.L.A.P..

### 3.3 Results

#### 3.3.1 Immunohistology of normal tissues

Serial sections of duodenum, liver and kidney were cut and stained either histochemically or by the indirect immunoperoxidase technique with NDOG2. There was no reactive staining with NDOG2, in any of these tissues but alkaline phosphatase activity was demonstrable histochemically on duodenal epithelium, liver sinusoids and kidney tubules. The NDOG2 antibody does not therefore cross react with these isoenzymes. In addition, no reactive staining could be demonstrated in any of the normal tissues listed in Table 3.2.

However, the following normal tissues did show positive reactive staining with NDOG2:-

##### a) Placenta

Reactive staining with the NDOG2 antibody could be demonstrated in placentae from 10 weeks gestational age. This staining was initially prominent around multinucleate syncytial buds but later spread to cover uniformly the villous syncytiotrophoblast plasma membrane (fig 3.1). There was slight staining of the syncytiotrophoblast basement membrane in the second and third trimester. The non-villous forms of trophoblast in the placental bed bound either none or very low quantities of NDOG2. Term chorionic membrane showed up to 50% of cytotrophoblast cells stained with NDOG2 and these were concentrated on the fetal aspect of the chorionic cytotrophoblast layer. NDOG2 showed reactive staining with all of the 50 term placentae examined.

b) Cervix

10 specimens of normal, healthy cervix were examined. There was some non-specific staining of endocervical gland secretions. This was reduced by the incorporation of normal human serum in the monoclonal antibody incubation, revealing specific NDOG2 binding to the luminal epithelial surface (Fig. 3.2) and some specific staining of gland secretions with NDOG2. The squamous epithelium of the ectocervix was consistently negative.

c) Endometrium

Samples of endometrium from 22 women in their reproductive years and from 3 women who were postmenopausal were examined. 13 samples were from the proliferative phase of the menstrual cycle, 8 were in the secretory phase and 1 patient was premenstrual. Reactive staining with NDOG2 was looked for on surface (Fig. 3.3) and glandular (Fig. 3.4) epithelium. Positive staining was always seen on at least some cells of the surface epithelium although it was sometimes absent from glandular epithelium in the same specimen (Table 3.3). Only a few glands were cut in longitudinal sections, but in those that were, some cells were always positive. There was reactive staining on the cell membrane with the strongest reaction occurring on the luminal surface of the cell in both epithelial types examined. The functional glandular epithelium showed a greater degree of reactive staining than the reserve cells but there was otherwise no obvious difference between cells expressing the NDOG2 determinant and those that did not.

21 from 22 specimens of premenopausal endometrium showed positive reactive staining with NDOG2. In 15 of these both glandular and surface epithelium was present but in 6 only glandular epithelium was seen. The negative specimen contained glandular epithelium (in cross section) alone.

Surface epithelium was present and positive in 15 sections, glandular epithelium was positive in 13 from 22 sections (Table 3.3). There was no variation in expression of the NDOG2 determinant throughout the cycle (Table 3.4) and the stromal tissue was consistently negative.

Reactive staining with NDOG2 was found in all 3 specimens of postmenopausal endometrium in both surface and glandular epithelium (Table 3.3).

d) Fallopian Tube

In all 4 samples studied there was reactive staining with NDOG2 on the luminal surface and of virtually all the epithelial cells (Fig. 3.5).

e) Lung

There was marked variation in reactive staining amongst the 6 specimens examined. In 3 specimens no staining was seen but in the remaining 3, specific staining presented as a linear streak on a single epithelial surface (Fig. 3.6) as opposed to circumscribing the cell, as might be expected from a typical cell membrane antigen. This pattern of expression may be consistent with enzyme secretion.



#### F) Thymus

The thymus gave the only example of cellular reactivity in normal tissues. A minor subpopulation of reticular cells in the thymic medulla gave a positive reaction which was diffuse throughout the cell, probably indicating cytoplasmic localisation of antigen. These cells comprised less than 1% of the cells in the thymus and were of typical reticular morphology, having a large pale staining nucleus and long cytoplasmic processes (Fig. 3.7).

#### 3.3.2 Immunohistology of gynaecological tumour tissue Ovarian Tumours

100 ovarian tumours were examined immunohistologically for the expression of the NDOG2 determinant, of these 44 were benign tumours and 56 malignant tumours.

Serial sections of one serous cystadenocarcinoma were stained by the immunoperoxidase technique with NDOG2 and histochemically for the enzyme alkaline phosphatase. The alkaline phosphatase reaction was intensely positive on precisely the same subpopulation of epithelial cells as bound detectable NDOG2 antibody. This enzyme activity was totally inhibited by 2mM L-phenylalanylglycylcine while 1mM levamisole gave only a marginal diminution in staining. These inhibiting properties are characteristic of the placental isoenzyme of alkaline phosphatase (Goldstein et al 1982). Stromal tissue showed a lesser amount of alkaline phosphatase activity localised on blood vessel endothelium which was inhibitable completely by 1mM Levamisole but not 2mM L-phenylalanylglycylglycine i.e. a typical inhibiting pattern of the tissue non-specific isoenzyme. NDOG2 did not bind to any stromal tissue.

25% of all benign tumours (Table 3.5) and 64% of malignant tumours (Table 3.6) showed variable degrees of reactive staining with NDOG2 but in no specimen examined were 100% of cells positive. In all specimens of primary ovarian cancers and benign cystadenomas examined, staining was present on the epithelial cell membrane (fig. 3.8 & 3.9); but secondary tumours, when positive, showed uniform distribution of staining throughout the cell. In both benign and malignant tumours it was the serous cystadenomas and cystadenocarcinomas that predominantly expressed P.L.A.P. (47% and 81% respectively). There was much variation in the degree of staining within each histological grade of tumour; however, when both primary and secondary tumour tissue was available from the same patient, the primary tumour usually showed the greater degree of reactive staining (Table 3.7 and figs 3.9 and 3.10). In all specimens the stromal tissue did not show specific reactive staining with NDOG2.

#### Endometrial Carcinoma

Specimens of endometrial cancer tissue were obtained from 17 patients. Of these, 2 were adenosquamous tumours and the remainder adenocarcinomas. The effect of histological type, stage and differentiation on the degree of staining was assessed. Both adenosquamous carcinomas were negative but 11 from 15 adenocarcinomas were positive. Intense reactive staining was seen on the cell surface of malignant cells with no intracellular or stromal staining (fig 3.11). The degree of staining was unaffected by stage or differentiation of the tumour (Tables 3.8 and 3.9 respectively).



### Cervical Carcinoma

12 squamous carcinomas of the cervix were examined, only 5 tumours showed reactive staining and this was, on the whole, scanty with 3 tumours showing reactive staining in less than 1% of the total number of cells and 2 with 1-25% of cells positive. The remaining 7 tumours were all negative. Again staining was confined to the cell membrane (fig 3.12).

### Other Gynaecological Malignancies

3 uterine sarcomas and 5 vulval carcinomas all failed to show reactive staining with NDOG2.

### 3.4 Conclusions and Discussion

Fresh frozen tissue was used both for immunohistological and histochemical examination, as formalin fixed, wax embedded tissues appear to loose the NDOG2 determinant. Positive reactive staining with NDOG2 was demonstrated in normal fallopian tube, endometrial and endocervical columnar epithelium, thymus, lung and placentae from 10 weeks gestation to term. Although, as far as I am aware, P.L.A.P. has not previously been described in association with fallopian tube epithelium, this distribution is the same as that described in the literature for P.L.A.P. (Fishman et al 1972, Nozawa et al 1980, Goldstein et al 1982). It has already been shown that NDOG2 fixes a P.L.A.P. determinant (section 2.3) and the absence of reactive staining on duodenum, kidney and liver is evidence that NDOG2 does not cross react with the other alkaline phosphatase isoenzymes.

Virtually all the specimens of endometrium examined showed positive reactive staining with NDOG2 and this was more marked on surface than glandular epithelium (Table 3.3) and this was unaffected by the stage of the menstrual cycle (Table 3.4). This is a much greater expression of P.L.A.P. by this tissue than has been previously described (Nozawa et al 1980) and suggests a greater sensitivity for immunohistology over those methods relying on the L-phenylalanine sensitivity of P.L.A.P. or heat stability.

No reactive staining with NDOG2 was demonstrated on the single specimen of testis examined although P.L.A.P. was described in association with this organ (Chang 1978) however the form of P.L.A.P. expressed is usually the rare 'D' variant (Goldstein et al 1982) a form not recognised by NDOG2.

Endocervical columnar endometrial and fallopian tube epithelia are all derived from the paramesonephric duct and all three express P.L.A.P.. Although no specimen of the rare fallopian tube carcinoma were available for study, both endometrial and cervical carcinomas express this enzyme. This suggests that there is an inbred inherence for tissues derived from similar regions embryologically and their tumours to express a particular antigen, in this case P.L.A.P., and that this is not due to a malignancy-induced change from the bone-liver-kidney isoenzyme to P.L.A.P. as suggested by Nozawa et al (1981). Although P.L.A.P. has been described in association with both endometrial and cervical cancer (Cadeau et al 1974, Kellen et al 1976) its expression by endometrial cancer does not appear to be affected by the stage of the tumour (Table 3.8) or the degree of differentiation (Table 3.9) as suggested by Kellen et al (1976).

In a similar vein all epithelial ovarian cancers derive from the so called germinal epithelium of the ovary which arises, embryologically, close to the paramesonephric duct, and a high proportion (64%) of such cancers studied showed positive reactive staining with NDOG2. Histochemistry performed on serial sections of tumour showed P.L.A.P. activity on the same subpopulation of cells as those with NDOG2. Serous cystadenocarcinomas showed a greater degree of specific staining than any other histological type studied (Table 3.6) and a similar distribution was seen when benign tumours were studied (Table 3.5). The histological grade of the tumour did not affect the degree of reactive staining with NDOG2, however the primary tumour usually showed a higher percentage of cells positive than did metastases from the same patient (Table 3.7).

Although P.L.A.P. is not tumour specific it is expressed by relatively few normal tissues and of these some (fallopian tube, endometrium and cervix) are usually removed at primary surgery for ovarian cancer. Expression of P.L.A.P. by thymus is minute and in any case tends to be intracellular and obviously expression by placentae is irrelevant when P.L.A.P. is considered as a tumour marker in ovarian cancer. The presence of P.L.A.P. in lung is potentially problematical, and as can be seen in Chapter 4 caused problems in interpreting raised serum P.L.A.P. levels in cigarette smokers. The reduced expression of this enzyme by tumour metastases was disappointing and could affect the sensitivity of P.L.A.P. as a tumour marker.

### 3.5 Tables, Charts and Figures

#### Legend for Figures

Positive reactive staining with NDOG2, using an indirect immunoperoxidase technique, counterstained with haematoxylin is demonstrated on frozen sections of the following tissues.

- |          |   |
|----------|---|
| Fig 3.1  | Term placenta.  |
| Fig 3.2  | Normal cervix.  |
| Fig 3.3  | Surface endometrial epithelium.   |
| Fig 3.4  | Glandular endometrial epithelium.   |
| Fig 3.5  | Fallopian tube epithelium.  |
| Fig 3.6  | Normal lung.  |
| Fig 3.7  | Normal thymus.  |
| Fig 3.8  | Ovarian serous cystadenoma showing grade 3 reactive staining.                   |
| Fig 3.9  | Primary ovarian serous cystadenocarcinoma, showing grade 4 reactive staining.   |
| Fig 3.10 | Omental secondary from the tumour in Fig 3.9 showing grade 2 reactive staining. |
| Fig 3.11 | Primary endometrial adenocarcinoma showing grade 4 reactive staining.           |
| Fig 3.12 | Primary cervical squamous carcinoma showing grade 1 reactive staining.          |



FIG 3.1

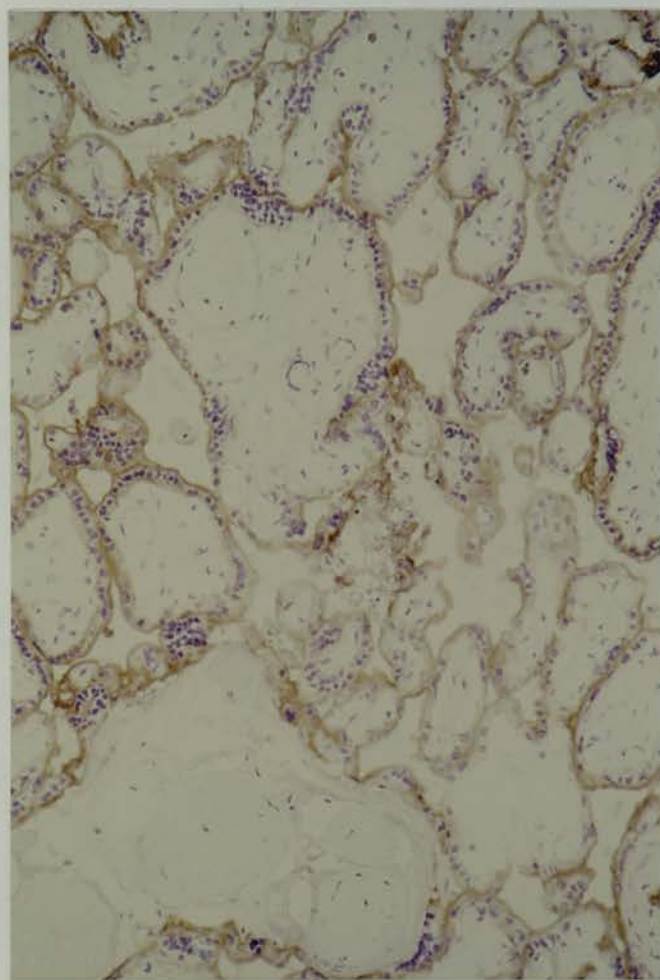


FIG 3.2

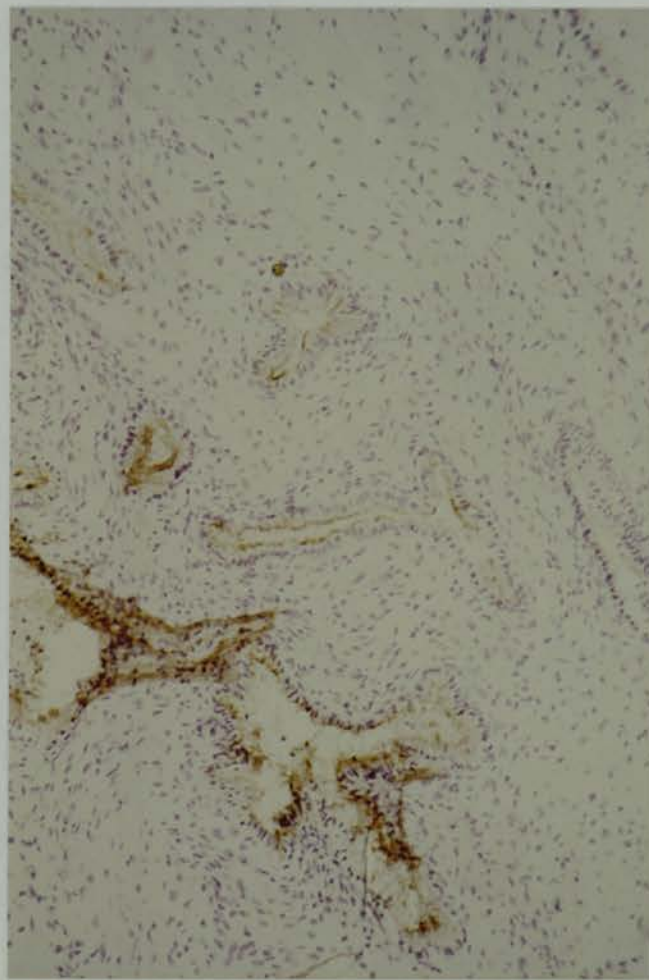


FIG 3.3

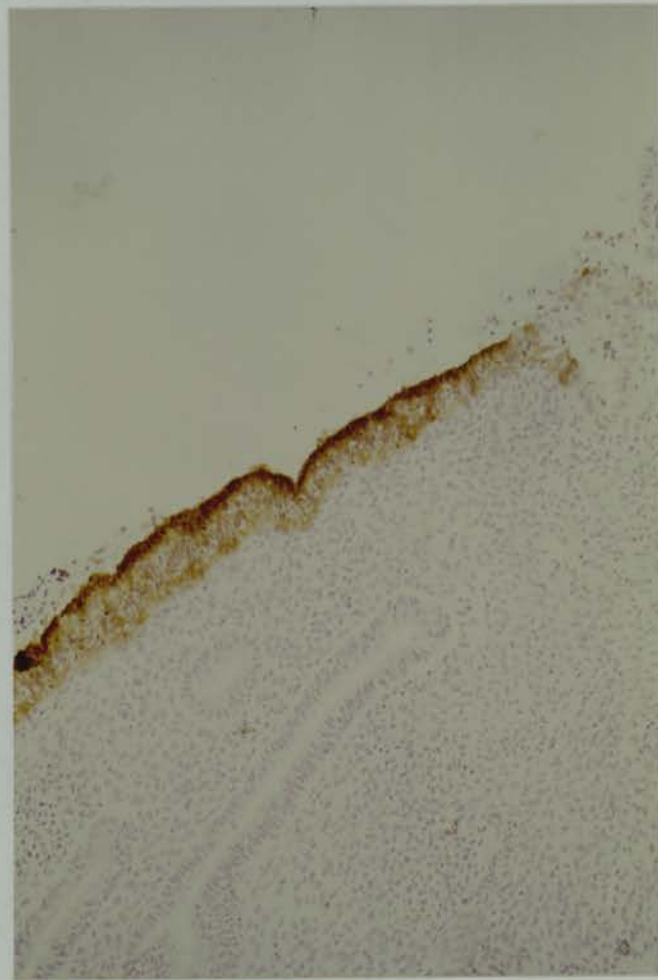


FIG 3.4

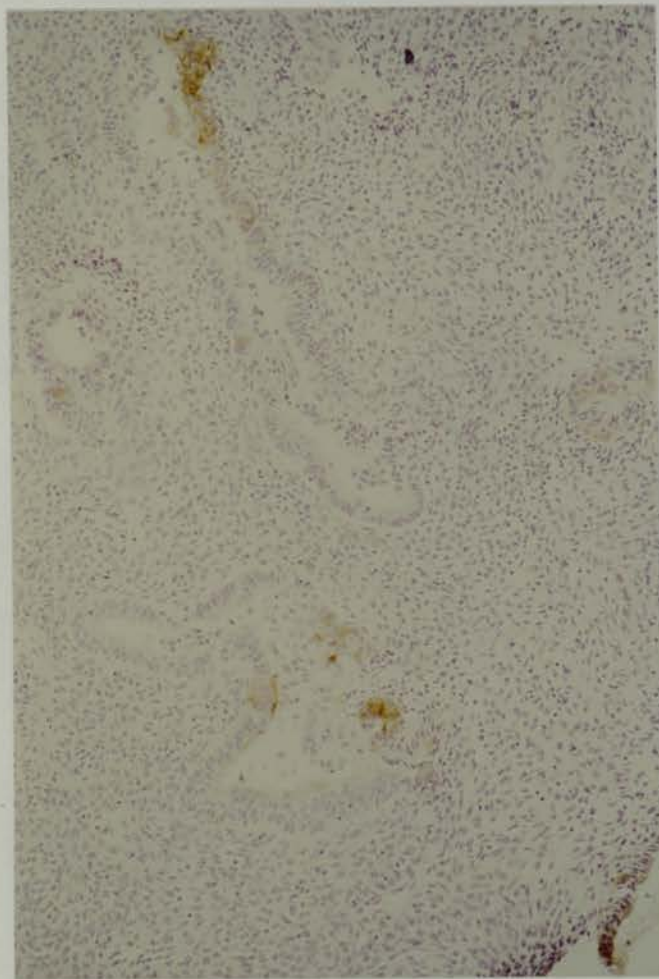


Fig 3.5

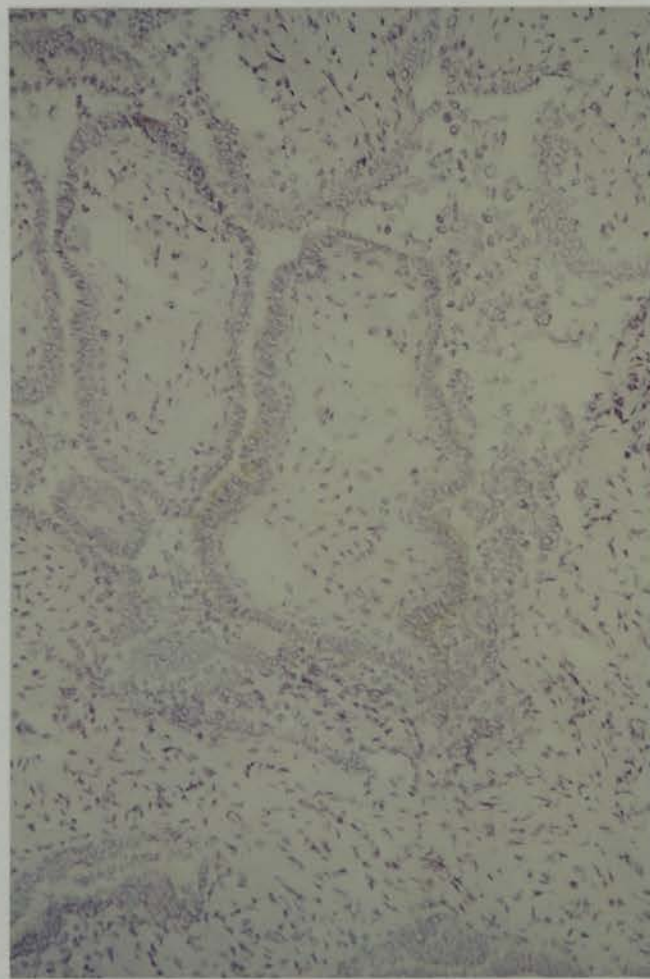


FIG 3.6

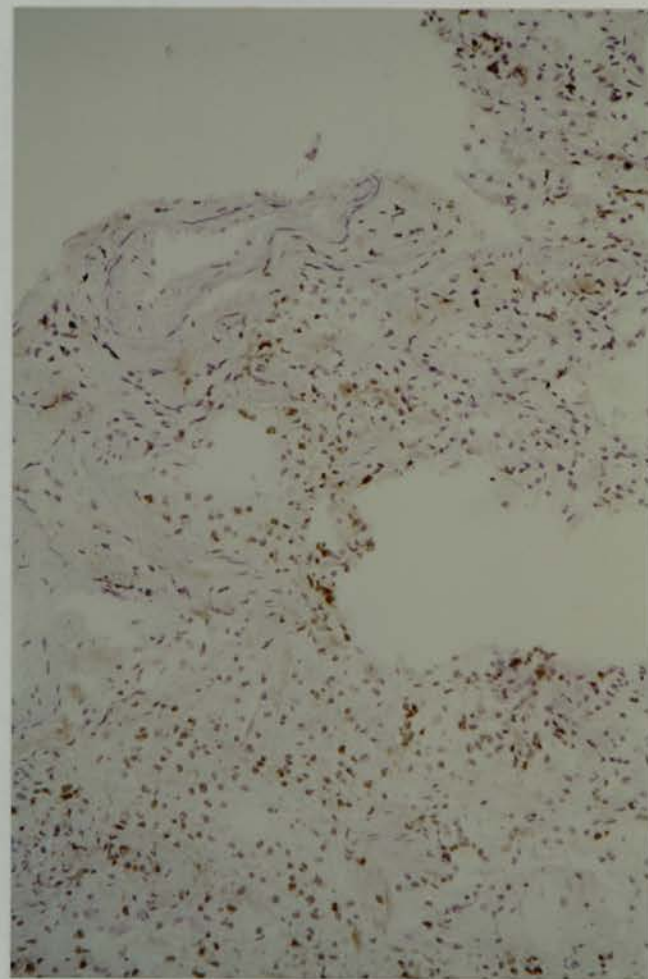




FIG 3.7

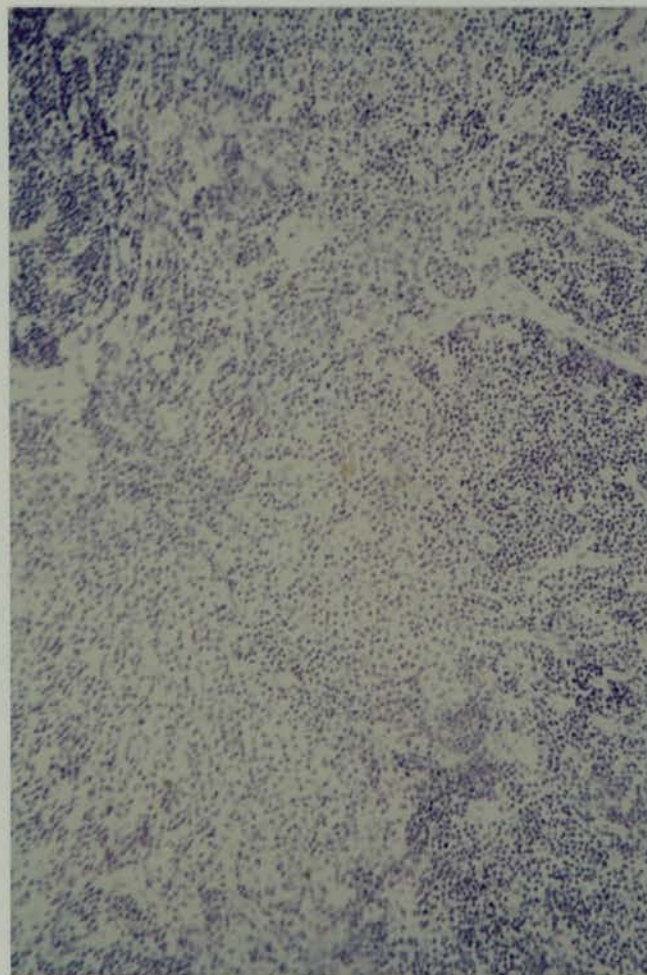


FIG 3.8

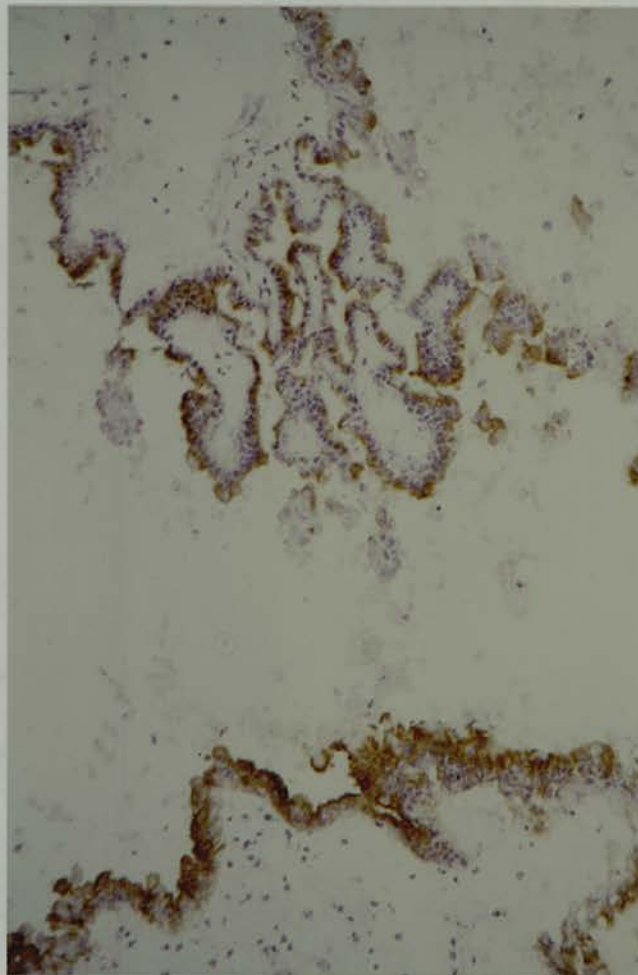


FIG 3.9

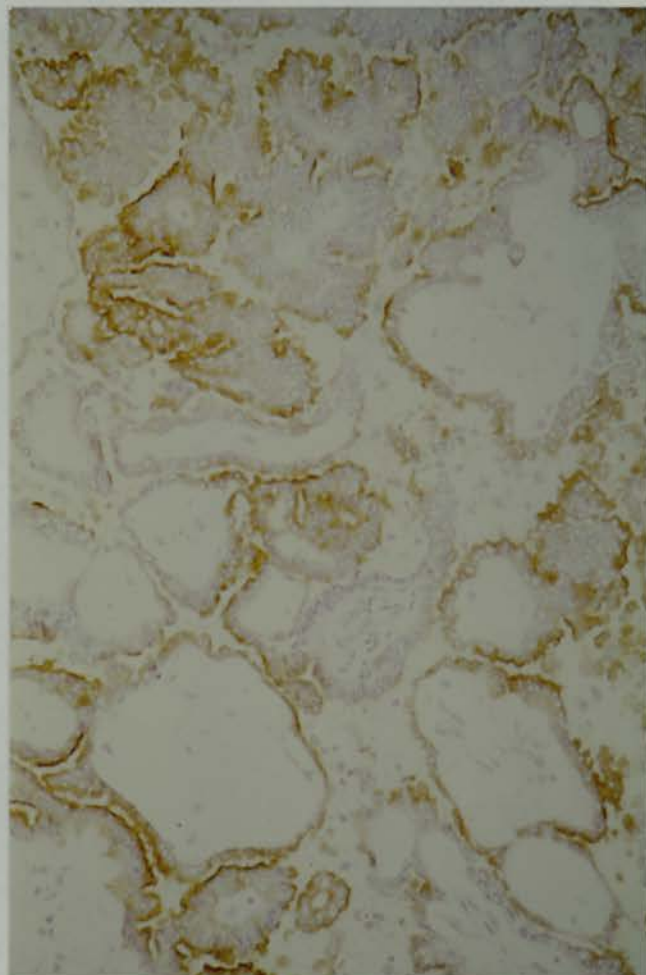


FIG 3.10

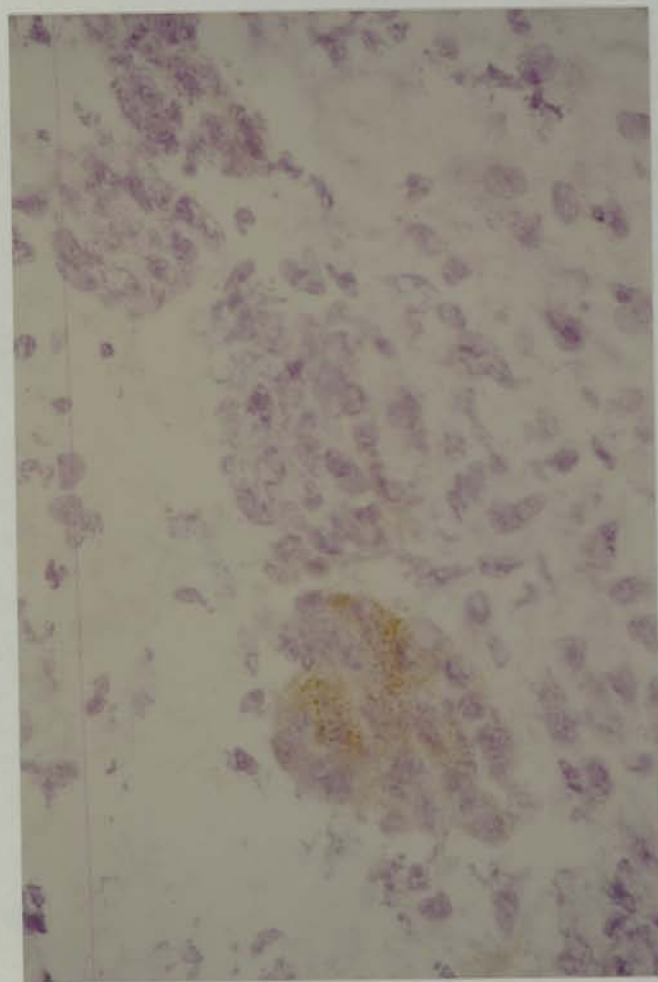


FIG 3.11

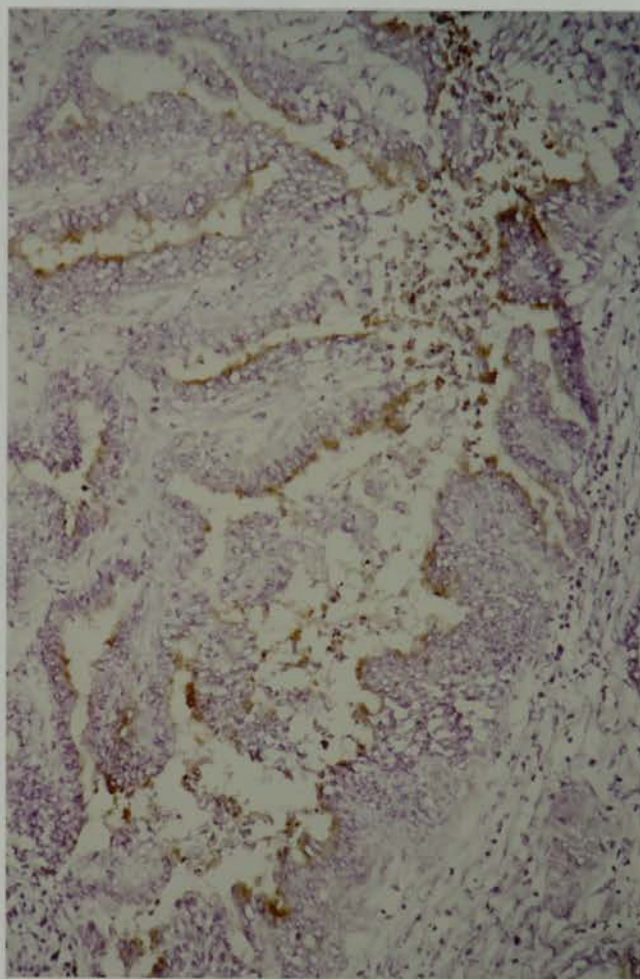


FIG 3.12

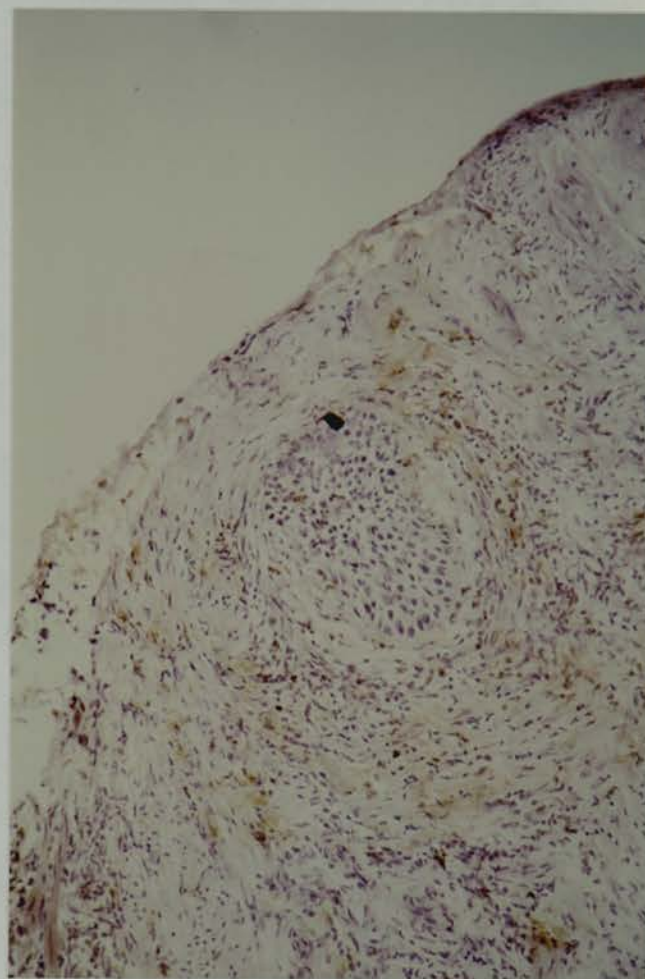




Table 3.1 Reactivity of monoclonal antibodies used in immunohistology experiments

Monoclonal Antibody	Immunoglobulin Type	Specificity	Antigen Distribution		Reference
			Tissue	Cellular Site	
NDOG2	Mouse IgG 2b	P.L.A.P.	This Chapter	This Chapter	This Thesis
MRC OX6 (Negative control)	Mouse IgG	Rat class II histocompatibility antigens	Absent from all human tissues	Membrane	McMaster & Williams (1979)
NDOG4 (Negative control)	Mouse IgG 1	Unknown	Human trophoblast and a small number of uncharacterised adult tissues	Cytoplasmic	In house MAB
BMH 5 (Negative control)	Mouse IgG 2	Unknown	Human trophoblast	Membrane	In house MAB
W6/32 (Positive control)	Mouse IgG 2	Human class 1 HLA antigens	Ubiquitous	Membrane	Branstable et al (1978)
TROMA 1 (Positive control)	Rat IgG	Intermediate filament protein	Differentiated structures including many epithelia	Cytoskeleton	Kemler et al (1981)
BMH 1 (Positive control)	Mouse IgM	Unknown	Ubiquitous	Membrane	In house MAB

Table 3.2     Reactive staining of normal human tissues with the NDOG2 antibody

Tissue	Reactivity	Number of specimens studied
Placenta	Positive	50 <sup>+</sup>
Cervix	Positive	10
Endometrium	Positive	25
Fallopian Tube	Positive	4
Lung	Weakly Positive	6
Thymus	Positive	1
Testis	Negative	1
Stomach	Negative	1
Duodenum	Negative	2
Jejunum	Negative	1
Ileum	Negative	1
Colon	Negative	2
Kidney	Negative	2
Ureter	Negative	1
Bladder	Negative	1
Adrenal	Negative	2
Thyroid	Negative	1
Parathyroid	Negative	1
Pituitary	Negative	1
Tonsil	Negative	2
Heart	Negative	2
Myometrium	Negative	4
Skin	Negative	1
Meninges	Negative	1

Table 3.3    The relationship between positive surface and glandular epithelium

Type of reactive staining with NDOG2 observed	Number of tissues examined			
	Proliferative Phase	Secretory Phase	Premenstual	Post-Menopausal
Positive surface with negative glandular epithelium	5	2	1	0
Positive surface and glandular epithelium	5	2	0	3
Positive glandular epithelium with non-surface epithelium seen	3	3	0	0
Negative glandular epithelium. No surface epithelium seen	0	1	0	0

Table 3.4 The degree of reactive staining with NDOG2 of samples of endometrium taken at different phases of the menstrual cycle

Epithelium	Degree of staining	Proliferative phase	Secretory phase	Premenstrual
SURFACE	Negative	0	0	0
	Grade 1	2	1	1
	Grade 2	4	0	0
	Grade 3	2	1	0
	Grade 4	2	2	0
GLANDULAR	Negative	5	3	1
	Grade 1	1	2	0
	Grade 2	7	2	0
	Grade 3	0	1	0
	Grade 4	0	0	0

The degree of staining was assayed as follows:

- Negative - No cells showing reactive staining with NDOG2
- Grade 1 - Less than 1% of cells showing reactive staining with NDOG2
- Grade 2 - 1-25% of cells showing reactive staining with NDOG2
- Grade 3 - 25-75% of cells showing reactive staining with NDOG2
- Grade 4 - 75-100% of cells showing reactive staining with NDOG2

Table 3.5 Degree of staining with NDOG2 compared with histological type of benign ovarian tumours (includes fimbrial cysts)

Histological Type	Degree of staining					Total
	Neg	G1	G2	G3	G4	
Serous cystadenoma	9	1	1	5	1	17
Mucinous cystadenoma	8	-	1	-	-	9
Fibroma	5	-	-	-	-	5
Dermoid cyst	5	-	-	-	-	5
Fimbrial cyst	-	1	1	-	-	1
Chocolate cyst	1	-	-	-	-	1
Theca granulosa cyst	1	-	-	-	-	1
Cystadenomafibroma	1	-	-	-	1	2
Follicular cyst	1	-	-	-	-	1
Thecalutein cyst	1	-	-	-	-	1
Thecoma	1	-	-	-	-	1

The degree of staining was graded as follows:

Grade 1 (G1) = Less than 1% of cells positive

Grade 2 (G2) = 1-25% of cells positive

Grade 3 (G3) = 25-75% of cells positive

Grade 4 (G4) = 75-100% of cells positive

Table 3.6 Degree of staining with NDOG2 compared with differentiation and histological type of ovarian carcinoma

Histological Type	Differentiation	Degree of staining with NDOG2					TOTAL
		Neg	G1	G2	G3	G4	
Serous	Borderline	1	-	-	1	-	33
	Well	2	-	5	-	4	
	Moderate	3	4	3	6	1	
	Poor	-	1	1	-	1	
Mucinous	Well	4	1	1	-	-	8
	Moderate	2	-	-	-	-	
	Poor	-	-	-	-	-	
Adenocarcinoma	Well	-	-	-	-	-	9
	Moderate	2	-	3	-	-	
	Poor	3	1	-	-	-	
Clear cell		1	2	-	-	-	3
Mixed Mullerian		-	1	-	-	-	1
Granulosa Cell		1	-	-	-	-	1
Malignant Teratoma		1	-	-	-	-	1

Table 3.7 Comparison of the degree of staining with NDOG2 between primary and secondary tumour in the same patient

Patient	Histology	Differentiation	Primary	Degree of staining with NDOG2 Secondary		
				Omental	Pelvic	Abdominal
13	Serous	Well	G4	G2	-	-
15	Adeno- carcinoma	Moderate	Negative	Negative	-	-
19	Serous	Moderate	G2	Negative	-	-
67	Serous	Moderate	Recurrence after primary surgery	G1	G1	-
71	Serous	Moderate	Negative	Negative	-	-
80	Mucinous	Well	G1	-	Negative	-
87	Mucinous	Well	Negative	G2	-	-
88	Serous	Moderate	G4	G2	-	-
98	Serous	Moderate	G2	G2	-	-
103	Serous	Well	G4	G2	-	-
104	Serous	Well	Negative	Negative	-	-

Table 3.8 The degree of staining with NDOG2 compared with the stage of endometrial carcinoma (adenocarcinoma)

Stage of tumour (FIGO)	Grade of staining				
	Neg	1	2	3	4
1	4	1	2	2	2
2			3		
3				1	
4					

Table 3.9 The effect of differentiation on the degree of reactive staining with NDOG2 in endometrial carcinoma (adenocarcinoma)

Differentiation	Grade of staining				
	Neg	1	2	3	4
Well differentiated	2	1	2		
Moderately differentiated	2		3	3	1
Poorly differentiated					1



## CHAPTER 4

### SERUM ASSAY

#### 4.1 Introduction

A serum assay measuring the presence and levels of a tumour marker which gave an accurate indication of the course of the disease would be very useful in the management of patients with ovarian cancer. This chapter describes the use of the NDOG2 antibody as a basis for such an assay.

NDOG2 recognises the enzyme P.L.A.P. and there are two basic ways of using antibodies to determine the levels of an enzyme:

- 1) Through the amount of enzymatic activity specifically bound by the antibody.
- 2) Through the amount of protein specifically bound by the antibody.

Assay 1 followed the first option whereas assays 2 and 3 attempted to measure the total protein bound. Enzyme linked immunosorbant assays (E.L.I.S.A.) were used in option 2 and not radioimmunoassays because of the proven sensitivity and convenience of the E.L.I.S.A. technique.

The discrepancies noted between option 1 and 2 may be due to the presence of inactive enzyme protein (measured in 2 but not in 1) or cross reaction of NDOG2 with some other serum protein. Attempts to distinguish between these were made by affinity chromatography and immunoblotting.

## 4.2 Patients

### Normal Controls

109 healthy female blood donors acted as controls for this study, 66 of these were non-smokers (mean age 39.4 years). The mean age of the 43 cigarette smokers was 35.4 years.

### Patients

#### a) With benign ovarian tumours

Preoperative serum samples were obtained from 6 non-smoking patients (mean age 52.4 years) whose tumours subsequently showed positive reactive staining with NDOG2.

#### b) With ovarian cancer

Serum samples were available from 52 patients with ovarian cancer. 44 patients (mean age 51 years) were followed up for between 4 and 26 months (mean 9.8 months); during this time 27 (61%) either died of their disease or developed a clinically obvious recurrence. Only 5 patients were cigarette smokers. Serum samples were collected at monthly intervals whilst the patient was undergoing chemotherapy and thereafter at 3 monthly intervals. All samples were spun down within 2 hours of collection and stored at  $-20^{\circ}\text{C}$  until use.

## 4.3 General Principles in running E.L.I.S.A.

### 4.3.1 Buffers used

a) Carbonate coating buffer - 1.59g  $\text{Na}_2\text{CO}_3$  (Analar) and 2.93g  $\text{NaHCO}_3$  (Analar) were made up to 1L in distilled  $\text{H}_2\text{O}$  (Analar) pH 9.6. This was stored at room temperature and was replaced every 2 weeks. 0.2g sodium azide was added to prevent bacterial growth.

b) P.B.S./Tween. This consists of reconstituted P.B.S. (oxoid) in distilled water to which 0.05% (volume by volume) Tween 20 was added.

c) Tris/citrate buffer consisting of 50mM Tris (Sigma) and 10mM citric acid (Sigma) pH 7.4 was used to store the trophoblast membrane extract (T.M.E.) used in assay 1. 0.05% Tween 20 was added for use in all washes in assay 1.

d) P.B.S. + 0.5% (weight by volume) bovine serum albumin (R.I.A. grade Sigma) with 2mMol Hepes pH 7.4 was the buffer used to dilute standard P.L.A.P. (Sigma) and antibodies used in assays 2 and 3 (unless otherwise stated).

#### 4.3.2 Blocking step

This was performed in assays 2 and 3 which used purified NDOG2, after coating with the first layer protein (P.L.A.P. or NDOG2 depending on the assay), using 10% (volume by volume) foetal calf serum (F.C.S.) in P.B.S. to block any remaining sites on the wells. This step was performed at room temperature over 2 hours. As 100ul of F.C.S. was used per well, evaporation was not a source of error and by incubation at room temperature adsorption of F.C.S. to the plates proceeds more rapidly allowing the assay to be completed that day.

This step was omitted from assay 1 where the F.C.S. present in NDOG2 supernatant would perform the same role.

#### 4.3.3 Incubations

With the exception of the blocking step and incubation with the appropriate substrate all other incubations were performed at 4°C. This ensured a constant temperature regardless of the season, for the reaction and ensures the protein remained stable. The substrate used in assay 1, paranitrophenyl phosphate (P.N.P.) was incubated overnight at 37°C while orthophenyldiamine (O.P.D.) was incubated at room temperature for 20 minutes.

#### 4.3.4 Substrates

O.P.D. was made up freshly immediately prior to use. 24.3ml of 0.1M citric acid and 25.7ml of 0.2M Na<sub>2</sub>HPO<sub>4</sub>, 2 H<sub>2</sub>O pH 5.0 were added to 50ml distilled water. O.P.D. was added in the ratio of 1mgm O.P.D. to 2.5ml of buffer, and an appropriate volume of substrate solution made up depending on the number of plates used. 4ul of 30% H<sub>2</sub>O<sub>2</sub> were added to every 10ml of solution immediately before 100ul of the whole was added to each well. The reaction proceeded for 20 minutes and was stopped with 50ul 2.5M H<sub>2</sub>SO<sub>4</sub>. The plates were read at 492nm on a titerteck multiskan spectrophotometer (Flow).

P.N.P. was made up in a 0.2M diethanolamine buffer. 100ul was added to each well and the plates incubated overnight at 37°C. The reaction was stopped with 3M NaOH and the optical density read at 405nm.

#### 4.3.5. Microtitrewell plates

These were Flow multiwell disposotray 96 U shaped non sterile well plates (Cat. No. 76/364/05) with flexible formed (non sterile) plate covers (Cat. No. 76/406/05). The edges of these plates were trimmed before they would

fit into the spectrophotometer. The plates were kept covered during all incubations at 4°C and room temperature and sealed when incubated at 37°C.

#### 4.3.6 Purification of the NDOG2 antibody

Whilst assay 1 relied on Rabbit anti mouse immunoglobulin to attach NDOG2 antibody, from spent supernatant, to the microlitre wells; in assays 2 and 3 purified NDOG2 antibody was adsorbed directly. NDOG2 was purified using Protein A affinity chromatography and sephacryl S300 column chromatography.

NDOG2 antibody was produced by bulk culture of the cell line in R.P.M.I 1640 with 2% F.C.S. (+g.p.s.).

##### a) Affinity Chromatography

An 8.5ml affinity column had previously been made by swelling protein A sepharose CL4B in P.B.S. (1gm will give 3.5ml of swollen gel with a capacity of 25mgm of protein per ml of swollen gel). Protein A interacts with the Fc portion of certain immunoglobulins including subclass IgG2b and will therefore remove these from a mixture of proteins.

The prepared column was first eluted with 50ml of 0.1M glycine/HCl pH 3.0 and then washed with a similar volume of P.B.S.. This step removed any immunoglobulin that might already be attached to the column and any sodium azide used to preserve the column when stored. All washes, elution steps and running the supernatant were performed under gravity. A convenient volume of supernatant, usually 500-600ml, was run through the column overnight; the rate

of flow was adjusted by varying the height between the column outlet and supernatant. The fractions of effluent were stored after each step and an aliquot run on electrophoresis, to check for NDOG2, at the end of the purification process.

After running the supernatant, the column was washed with 50ml each of P.B.S., P.B.S. + 6% NaCl and more P.B.S. prior to elution with 0.1M glycine/HCl pH 3.0. 1ml fractions were collected and read in a spectrophotometer, previously blanked with 0.1M glycine/HCl buffer, at 280nm. Protein was eluted between fractions 3 and 9 inclusive, which were immediately neutralised with concentrated Tris (pH6.8) and the samples pooled.

b) Sephacryl S300 column chromatography

A previously loaded 1.5cm x 74.5cm column containing 365.5ml of swollen sephacryl S300 was prepared prior to running the eluate. All air bubbles were excluded from the system and the column washed with 500ml P.B.S. pumped from top to bottom at 25ml per hour.

The eluate was pumped into the column, followed by P.B.S. and 5ml fractions collected. The optical density of each fraction was read in a spectrophotometer, previously blanked with P.B.S., at 280nm. An elevated O.D. was recorded between fraction 43 and 47 inclusive. These samples were pooled.

c) Calculation of amount of protein collected

Immunoglobulins give an absorbance of 1.4 (at 280° A) at a concentration of 1mg/ml. ∴mg of immunoglobulin = 
$$\frac{\text{sum of O.D.} \times \text{No. of mls of pooled sample}}{1.4}$$

In this example mg of NDOG2 = 
$$\frac{0.148 \times 24}{1.4} = 2.54\text{mgm}$$

∴ We have 2.54mgm in 24ml or a concentration of 0.106mg/ml of NDOG2.

d) Concentrating NDOG2

In this thesis purified NDOG2 has been used in 3 ways:

- a) In serum assays 2 and 3;
- b) In making the NDOG2 column;
- c) In R.I.S..

for a & b NDOG2 was concentrated to 3.5mg/ml and for c to 2.6mg/ml. The method was the same for both.

NDOG2 was concentrated using an amnicon filtration unit. A PM 30 ultrafiltration membrane which allows substances of molecular weight of less than 30,000 to pass and which can withstand pressures of up to 75lb/sq. in. was used. NDOG2 was loaded and the unit placed on a magnetic stirrer to stop aggregation of protein on the membrane. A filtration pressure of 25lb/sq. in. of Nitrogen was used and the filtrate collected, as a precaution, in case the membrane ruptured. The volume that the preparation was to be concentrated down to was calculated using the formula:



Final volume =

$$\frac{\text{Concentration of NDOG2 in sample}}{\text{Concentration of NDOG2 desired}} \times \text{volume of sample}$$

The antibody was stored at  $-20^{\circ}\text{C}$  until use.

#### e) Electrophoresis

The purity of the antibody in each batch was demonstrated by polyacrylamide gel electrophoresis of a small sample which was compared to that of a standard NDOG2 preparation of known purity. The method of gel electrophoresis is described in section 4.4.4.

### 4.4 Serum Assay 1

#### 4.4.1 Introduction

This assay is based on a method initially described by McLaughlin et al (1983) for use with a similar monoclonal antibody. The NDOG2 is bound to the microlitre plate wells by means of a rabbit anti mouse (R.A.M.) immunoglobulin. NDOG2 will bind any P.L.A.P. which in turn catalyses the hydrolysis of paranitrophenyl phosphate and the resultant colour change is measured by means of a spectrophotometer (fig 4.12).

Term placentae are a convenient source of P.L.A.P. and a method of extracting this and calculation of its activity is described before the assay method. Placentae may contain other alkaline phosphatases but as NDOG2 binds P.L.A.P. alone only this isoenzyme can be measured.

This assay depends on the enzyme activity of the antigen being measured, the other two assays do not.

#### 4.4.2 Preparation of Trophoblast Membrane Extract (T.M.E.)

This method was adapted from that originally described by Smith et al (1974) and Standring and Williams (1978).

A term placenta was obtained from the Central Delivery Suite within 30 minutes of delivery, and the cotyledons cut into small pieces. The amnion, chorion blood vessels and umbilical cord were discarded. The cut cotyledons were transferred to a sieve and washed with 2l 0.1M  $\text{CaCl}_2$  (Analar) and 1l P.B.S. (Oxoid), shredded in a mincer and suspended in 350ml of 0.15M NaCl (Analar). This suspension was stirred for 30 minutes at  $4^\circ\text{C}$  to separate the chorionic villi from the bulk of placental tissue. The mixture was sieved, the fluid collected and centrifuged at 3000 r.p.m. for 10 minutes, in an MSE 18, at  $4^\circ\text{C}$ . The supernatant was recentrifuged at 9200 r.p.m. for 10 minutes and finally at 18000 r.p.m. for 90 minutes. The pellet containing trophoblast membrane was saved and resuspended in 10ml P.B.S.. 25ml of 6% Triton X (B.D.H.) in P.B.S. (volume by volume) was added and the mixture transferred to a Damps Homogeniser. After homogenisation (10 strokes) the solution was stirred for  $\frac{1}{2}$  hour at  $4^\circ\text{C}$  and then centrifuged for 90 minutes at 18000 r.p.m. at  $4^\circ\text{C}$ . The supernatant was transferred to a dialysis bag, which had been boiled for 10 minutes and checked for leaks. This was suspended in a conical flask containing 50mM Tris (Sigma), 10mM citric acid (Sigma) pH 7.4 at  $4^\circ\text{C}$ .

The Tris/citrate buffer was changed 3 times over 36 hours. The contents of the dialysis bag then contained P.L.A.P. in a Tris/citrate buffer and were aliquoted and stored at  $-20^\circ\text{C}$  until use.

#### 4.4.3 Calculation of the activity of P.L.A.P. in the T.M.E. preparation

The T.M.E. preparation was diluted to 1 in 30 and 1 in 100 in a 0.2mol/l diethanolamine buffer (Sigma). 0.1ml of each dilution was added to 3ml of paranitrophenol phosphate in the same buffer and the optical density read in a 1cm cuvet immediately and after 3 minutes at 405nm. The spectrophotometer had initially been blanked with 3.1ml of substrate.

P.L.A.P. (Sigma) which contains 116U/L was reconstituted with 3ml of buffer and was used in a similar experiment, both neat and in a 1 in 2 dilution, as a control. The level of activity quoted for this preparation was only valid for up to 3 hours after reconstitution.

An International unit of alkaline phosphatase is that amount of enzyme which will convert 1 micromole of substrate per minute per litre of test solution under specified conditions (30°C) and may be calculated using the following equation:

International Units per litre (I.U./L) =

$$\frac{\Delta A \text{ per 3min} \times 1000 \times 3.1 \times \text{T.C.F.}}{18.75 \times 0.1 \times 3}$$

Where  $\Delta A$  per 3 minutes = Final A (O.D. after 3 minutes) - Initial A (O.D. at start).

T.C.F. = Temperature conversion factor.

1mM of paranitrophenol in 1 litre absorbs 18.75 O.D. units at 405nm (Sigma Bulletin)  $\therefore$  1 micromole/ml will also absorb 18.75 O.D. units. As the total volume is 3.1ml (3ml substrate, 0.1ml test solution) 1 micromole in 3.1ml will absorb  $\frac{18.75}{3.1}$  O.D. units.

The change of absorbance ( $\Delta A$ ) observed represents an increase of  $\frac{\Delta A \times 3.1}{18.75}$  umole paranitrophenol.

$$\therefore \text{I.U./0.1ml} = \frac{\Delta A \times 3.1}{\text{No. of minutes} \times 18.75}$$

$$\therefore \text{If we read for 3 minutes I.U./0.1ml} = \frac{\Delta A \times 3.1}{3 \times 18.75}$$

$$\begin{aligned} \therefore \text{I.U./L} &= \frac{\Delta A \times 3.1 \times 1000}{3 \times 18.75 \times 0.1} \quad \text{At the temperature of the experiment.} \\ &= \frac{\Delta A \times 3.1 \times 1000 \times \text{T.C.F.}}{3 \times 18.75 \times 0.1} \\ &= \Delta A \times 551 \times \text{T.C.F.} \end{aligned}$$

This experiment was performed at 27°C giving a T.C.F. of 1.13 and the T.M.E. had previously been heated to 65°C for 10 minutes.

1) At at 1/30 dilution of the T.M.E.

$$\Delta A = 0.988 - 0.138 = 0.850$$

$\therefore$  P.L.A.P. activity at 1/30 dilution =

$$0.85 \times 551 \times 1.13 = 529.14 \text{ I.U./L}$$

$\therefore$  Neat T.M.E. contains 15,877 I.U./L

2) At a 1/100 dilution of the T.M.E.

$$\Delta A = 0.272 - 0.222 = 0.250$$

∴ P.L.A.P. activity at 1/100 dilution =

$$0.25 \times 551 \times 1.13 = 155.66$$

∴ Neat T.M.E. contains 15,566 I.U./L

The average of these two results = 15,721 I.U./L.

A parallel control experiment was performed using a standard sigma preparation of known activity. The results obtained (118 I.U./L) correlated well with this preparation's known activity (116 I.U./L).

#### 4.4.4 Assay Method

100ul of a 1 in 1000 dilution of a rabbit anti mouse immunoglobulin (DAKO) in carbonate coating buffer was added to each well and the plates incubated overnight. The wells were washed three times with Tris/citrate buffer, 0.05% Tween 20, incubated for 1 hour with 100ul of a 1 in 10 dilution of spent NDOG2 supernatant and then washed three times. 100ul of either test serum (neat or in a 1 in 10 dilution in Tris/citrate buffer) or serial dilutions of 1 in 3000 or 1 in 5000 dilution (depending on the batch used) of T.M.E. in Tris/citrate buffer was added to each well and incubated for 1 hour. The wells were washed 3 times and 100ul of P.N.P. added to each well; the plates were sealed and incubated overnight at 37°C. The reaction was stopped with 50ul of 3M NaOH and the optical density (O.D.) read at 405nm using a Tietertek multiskan spectrophotometer (Flow).

All samples were tested in triplicate and an average of the O.D. taken. A standard curve was constructed using the serial dilutions of T.M.E. from which the quantity of P.L.A.P. in each sample of patient's serum was obtained.

This method differs in a number of ways from that described by McLaughlin et al (1983):

1) NDOG2 supernatant and R.A.M. were each titrated, with other assay parameters constant, to find the lowest concentration of each consistent with maximum sensitivity. R.A.M. at a concentration of 1 in 1000 (as opposed to 1 in 200 (McLaughlin et al 1983)) and NDOG2 supernatant at a concentration of 1 in 10 filled these parameters.

2) T.M.E. was used to make the standard curve as opposed to commercially available P.L.A.P.. This titration curve showed a linear response as did serial dilutions of malignancy serum (fig 4.2).

3) Washes were performed using Tris/citrate buffer +0.05% Tween 20 as opposed to P.B.S./Tween.

T.M.E. was initially stored in P.B.S. and it was noted that its P.L.A.P. activity dropped over a period of several months. To test the hypothesis that P.B.S. was causing product inhibition of the enzyme, T.M.E. was diluted to 1 in 30 and 1 in 100 in Tris/citrate buffer and each divided into 2 samples. The first samples were incubated with P.N.P. and the O.D. measured immediately and then at



30 second intervals for 3 minutes. The second samples were left for 30 minutes to allow any phosphate to be released and then its activity similarly measured, a greater than 10 fold increase in activity was demonstrated (fig 4.1). Thereafter, T.M.E. was stored in Tris/citrate buffer without loss of activity.

#### 4.4.5 Results

##### a) Calculation

A graph of O.D. against serial dilutions of T.M.E. was plotted. The T.M.E. preparation was initially diluted from the original preparation to 1 in 3000 so that each 1/10 dilution of this preparation was equivalent to 0.5 I.U. P.L.A.P. activity. The reaction proceeded in a straight line initially, levelling off at higher concentrations of T.M.E. (fig 4.2) where the O.D. of the neat test serum fell on the straight portion of the graph then this value was read off. If it occurred on the flattened portion then the 1 in 10 dilution was read and multiplied by a factor of 10.

##### b) Normal range

###### i) Non smokers

McLaughlin et al (1983) quote an upper limit of normal for their assay of 0.1 I.U./L. Using this level 4 out of 66 volunteers had levels above this (2 of 0.15 I.U./L and 2 of 0.25 I.U./L). Therefore, an upper limit of normal for this assay was taken as 0.25 I.U./L.

(ii) Smokers

Serum was obtained from 43 volunteers who were cigarette smokers and all were asked how many cigarettes a day they smoked. 17 smokers had P.L.A.P. levels of less than 0.25 I.U./L and in 14, P.L.A.P. levels were less than 0.1 I.U./L. The total number of cigarettes smoked a day was correlated with serum P.L.A.P..

1-10 cigarettes	18 cases	range 0-3.7	(mean 0.52) I.U./L
11-20 cigarettes	16 cases	range 0-2.95	(mean 1.07) I.U./L
21+ cigarettes	9 cases	range 0.3-0	(mean 1.24) I.U./L

The mean levels of P.L.A.P. increased with the number of cigarettes smoked a day but, interestingly, the person with the highest serum level (0.37 I.U./L) only admitted to smoking 10 cigarettes a day. As patients are often inaccurate when admitting the number of cigarettes they smoke, due caution should be taken in interpreting elevated serum P.L.A.P. levels in such cases. In the event only 5 patients with ovarian cancer in this study were known cigarette smokers.

c) Serum half life of P.L.A.P.

Serum levels of P.L.A.P. were estimated, preoperatively and at daily intervals for 10 days after operation from a non cigarette smoking patient who had a stage 1a (i) ovarian carcinoma completely resected. The half life of P.L.A.P. in vivo was 4 days (fig 4.3) which is in accord with that reported in the literature (Fishman 1974).

d) Benign ovarian tumours

Preoperative serum P.L.A.P. levels were undetectable in 5 out of 6 patients with benign ovarian tumours and 0.21 I.U./L in one (Table 4.1).

e) Ovarian carcinoma

This section attempts to answer the following questions:

- i) Do preoperative serum enzymically active P.L.A.P. levels correspond with the immunohistology of the tumour?

Preoperative serum samples were obtained from 20 patients in whom fresh tumour tissue was available for immunohistological examination. All these patients denied cigarette smoking and in 12 there was positive reactive staining with NDOG2 (4 grade 4, 2 grade 3, 4 grade 2 and 2 grade 1).

Serum P.L.A.P. levels were elevated in all patients with greater than grade 1 reactive staining with the exception of patient 2 (Table 4.2). This patient had a stage 2c serous cystadenocarcinoma of borderline malignancy which showed grade 3 reactive staining but whose serum P.L.A.P. levels were normal. Conversely a patient (No. 36) with negative immunohistology had elevated serum P.L.A.P. and this subsequently proved to be a good tumour marker, accurately reflecting the course of her disease. It was possible that an unrepresentative sample of tumour was initially examined in this case.

The degree of reactive staining and elevated preoperative serum P.L.A.P. showed close correlation as one would expect. Thus, those tumours in whom over 25% of cells expressed this antigen (grade 3 and 4 reactive staining) and excluding the tumour of borderline malignancy, the

range of values was between 0.45 and 8.5 I.U./L with a mean of 4.54 I.U./L compared with a range of 0.95-2.1 I.U./L (mean 1.59 I.U./L) in tumours with grade 2 reactive staining and of less than 0.1 I.U./L in 9 out of 10 tumours with less than 1% of their cells showing positive staining (Table 4.2).

ii) Is enzymically active P.L.A.P. a good tumour marker for ovarian cancer?

The upper limit of normal for this assay was taken as 0.25 I.U./L. The effect of lowering this to 0.1 I.U./L in those patients in whom P.L.A.P. proved to be a poor marker did not improve the predictive value of this assay.

24 patients were followed from before primary surgery, 17 patients were recruited shortly after surgery but all had residual disease present and 3 were recruited while in remission. Fresh tumour tissue was available for immunohistology in 27 cases. During this study 30 patients (68%) either developed an obvious tumour recurrence or died of their disease.

The serum levels of enzymically active P.L.A.P. rose or fell appropriately, reflecting the course of the disease, in 12 cases (27%) but was of poor predictive value in the remaining 32 patients. The results of this assay are summarised in Tables 4.3 and 4.4. In 6 out of the 12 cases (patients 13, 20, 24, 38, 39 and 40) there were falls of serum P.L.A.P. levels corresponding to the degree of surgical debulking achieved, and subsequent falls in levels

during chemotherapy. These patients remain well with normal serum P.L.A.P. levels (e.g. fig 4.4). In the other six cases (patients 22, 23, 25, 36 and 43) there were initial falls in serum P.L.A.P. levels corresponding to surgery and chemotherapy with subsequent rising levels reflecting proven tumour recurrence (e.g. fig 4.5). Tumour tissue was available in 11 cases, 9 of which showed grade 2 or greater reactive staining with NDOG2.

In 23 cases serum P.L.A.P. was never elevated but in the remaining 9 cases P.L.A.P. was elevated at some stage of the disease. However the levels did not totally accurately reflect the course of the disease. Four distinct patterns were noted in these patients.

a) P.L.A.P. only rose prior to the patient's death, in the presence of massive recurrent disease, in one case (patient 34). No preoperative serum sample was available from this patient but her primary tumour showed grade 2 reactive staining with NDOG2.

b) P.L.A.P. levels returned to normal levels after primary surgery in 2 cases (patients 6 and 32), even though residual tumour remained. Both patients primary tumours showed grade 4 reactive staining with NDOG2 and in patient 6 the primary tumour was incompletely excised. In the case of patient 32 while the primary tumour was excised metastases, showing grade 2 staining, remained. Both these patients failed to respond to chemotherapy but serum P.L.A.P. remained in the normal range (e.g. fig 4.6).

c) In 4 patients serum P.L.A.P. levels fell inappropriately, in 3 cases (patients 15, 19 and 44) P.L.A.P. levels initially reflected the course of the disease but then fell even though there was continued massive tumour growth (e.g. fig 4.7). In one case (patient 12) P.L.A.P. levels fell following surgery even though the patient ultimately died of her disease. Tumour tissue was available for immunohistological examination only in patient 19, and showed grade 4 reactive staining. This inappropriate fall in levels of P.L.A.P. may reflect the death of the P.L.A.P. producing cell line with chemotherapy but the continued growth of other cell lines. Alternatively, chemotherapy may be affecting the ability of the cell to produce enzymically active P.L.A.P..

d) Elevated serum P.L.A.P. due to cigarette smoking was seen in 2 cases (patients 18 and 45). Patient 18, whose tumour showed grade 3 reactive staining was recruited while in remission; she subsequently remained well, with no additional therapy, although her serum P.L.A.P. levels fluctuated (fig 4.8). Patient 45 was a heavy smoker whose serum levels remained elevated prior to and following surgery and chemotherapy. Both patients remain well 17 and 5 months respectively after treatment finished.

iii) Does the degree of reactive staining with NDOG2 act as a reliable guide as to those patients who should be followed up using this assay?

Immunohistological examination of fresh tumour tissue was performed in 26 patients and compared with the predictive value of this assay (Table 4.3). In the 15 cases where



the tumour showed grade 2 or greater reactive staining with NDOG2, in a patient who was a non smoker, this assay was of predictive value in 9 cases. However as described in the previous section in 2 cases (patients 6 and 32) it did not reflect the course of the disease; in one case (patient 34) P.L.A.P. only became elevated with gross recurrent disease and in a further instance (patient 19) fell inappropriately. Serum P.L.A.P. was always within the normal range in one patient with a serous cystadenocarcinoma of borderline malignancy (patient 2).

In only one (patient 22) out of 6 cases with grade 1 reactive staining did serum P.L.A.P. levels reflect the course of the disease as it did with one patient with negative staining (patient 36).

### Conclusions

Overall (all patients studied) enzymically active P.L.A.P. as measured by this assay, proved to be a disappointing tumour marker. However, in those cases where immunohistological examination of tumour tissue had been performed and grade 2 or greater reactive staining noted, then this assay predicted the course of the disease in 60% of cases. Nonetheless in five patients in particular (patients 2, 6, 19, 32 and 34) where, based on the immunohistology, this assay might reasonably be expected to accurately reflect the course of the disease, it failed to do so.

There are 3 possible reasons for this:

- 1) The assay is not sufficiently sensitive;
- 2) In any one tumour there are several different cell lines, only one of which may produce P.L.A.P.. This cell line may be preferentially destroyed by chemotherapy, or have a low potential for metastasis in some cases;
- 3) P.L.A.P. may be produced in an inactive form by certain tumours. This may be due to chemotherapy damaging the pathway producing P.L.A.P. resulting in inactive enzyme fragments being produced.

To test hypotheses 1 and 3 further assays were developed in an attempt to measure the total protein recognised by NDOG2, independent of enzymic activity.

#### 4.5 Serum Assay 2

##### 4.5.1 Introduction

This modified indirect E.L.I.S.A. (Voller et al 1976) was used to see if NDOG2 could recognise P.L.A.P., present in human serum, when adsorbed directly to the E.L.I.S.A. wells.

##### 4.5.2 Method

Each half of 2 E.L.I.S.A. plates were coated, in duplicate, with 100ul/well of 24 halving dilutions of P.L.A.P. (Sigma) (10mgm/ml to zero) in 10% normal human serum in carbonate buffer, overnight at 4°C. 100ul of neat (750ug/ml) 1 in 4 or 1 in 16 dilutions of NDOG2 were added to each well of the appropriate half plate and buffer (P.B.S./B.S.A./Hepes) alone added to the final half plate. The plates were

incubated for  $1\frac{1}{2}$  hours at  $4^{\circ}\text{C}$ , washed and then incubated for  $\frac{1}{2}$  hour with 100ul of a 1 in 200 dilution of peroxidase conjugated rabbit anti mouse immunoglobulin (Dako) at  $4^{\circ}\text{C}$ . The reaction was developed with O.P.D..

#### 4.5.3 Results

There was no difference between background optical density (that generated by the half plate incubated with P.B.S./B.S.A./Hepes alone) and those of the other half plates, nor was there any titration.

#### 4.5.4 Conclusions

Because of the background problems, this assay was abandoned in favour of assay 3.

### 4.6 Serum Assay 3

#### 4.6.1 Introduction

This is a modified double sandwich E.L.I.S.A. (Voller et al 1976). P.L.A.P. is captured from test samples by NDOG2 directly adsorbed to microtitre plate wells, Rabbit anti human P.L.A.P. polyclonal antiserum (Dako) is used as second layer antibody and, as this is not supplied in a peroxidase conjugated form, a peroxidase conjugated goat anti rabbit immunoglobulin (Miles) was used prior to development with O.P.D. (fig 4.13).

Rabbit anti human P.L.A.P. was used as the second layer antibody (as opposed to first layer) to avoid blocking its active sites with any intestinal alkaline phosphatase present in test sera, and so that the NDOG2 determinant on P.L.A.P. is not bound by this polyclonal antiserum.

#### 4.6.2 Development of this assay

##### Experiment 1

The wells of each half of 2 E.L.I.S.A. plates were coated with 50ul of either 10ug/ml, 50ug/ml, 100ug/ml or 200ug/ml of NDOG2, overnight at 4°C. The wells were blocked with 10% F.C.S. and 50ul of 24 serial, doubling, dilutions from 10mg/ml P.L.A.P. (Sigma) to zero were incubated in duplicate at 4°C for one hour. The plates were washed and 50ul of a 1 in 150 dilution of rabbit anti human P.L.A.P. (Dako) added to each well and incubated at 4°C for ½ hour. The plates were again washed and 50ul of a 1 in 200 dilution of a peroxidase conjugated goat anti rabbit immunoglobulin added, and incubated at 4°C for ½ hour. The plates were developed with O.P.D..

##### Results (Experiment 1)

These are summarised in fig 4.9. The wells that had been coated with 50ul 200 ug/ml, 100ug/ml and 50ug/ml of NDOG2 gave over-range readings for too many dilutions of P.L.A.P. to be of use but the wells coated with 10ug NDOG2 gave a favourable titration. However, the background was rather high in this assay. This was unlikely to be due to rabbit anti human P.L.A.P. cross reacting with bovine intestinal alkaline phosphatase present in F.C.S. as this antibody only cross reacts with the human form of this isoenzyme (Dako Data Sheet). A more likely reason for this high background, supported by the development time of only 3 minutes, was that the concentrations of second and third layer antibody were too high.

### Experiment 2

This was performed to try and reduce background readings and to see if a 50ul coating concentration of 20ug/ml or 5ug/ml NDOG2 would give a better titration than 10ug/ml. Experiment 1 was repeated using these concentrations of NDOG2 to coat the wells and using the second layer antibody in a 1 in 250 dilution and the third layer antibody in a 1 in 1000 dilution.

### Results (Experiment 2)

These are summarised in fig 4.10. A coating concentration of 10ug/ml NDOG2 gives the best titration and the background optical density readings were reduced to a satisfactory 0.4.

As in assay 1, malignancy sera showed a linear titration when used in this assay.

#### 4.6.3 Method of Serum Assay 3 used

Microlitre wells were each coated with 50ul of 10ug/ml NDOG2 in carbonate buffer, overnight at 4°C. The wells were blocked by incubation with 100ul of 10% F.C.S. for 2 hours at room temperature. A calibration line of 18 serial dilutions of P.L.A.P. (Sigma) from 10mg/ml to zero was incubated in duplicate. This occupied the first 3 rows of the plate, leaving room for 30 serum samples (in duplicate) to be tested, one of which was always from a healthy, non smoking control. 50ul of either test serum or standard P.L.A.P. solution was added to each well and incubated at 4°C for 1 hour and the plates washed in P.B.S./Tween 3 times. 50ul of rabbit anti human P.L.A.P.

antiserum (Dako) in a 1 in 250 dilution was added to each well and incubated at 4°C for ½ hour. The plates were again washed 3 times and incubated for a further ½ hour at 4°C with 50ul of a 1 in 1000 dilution of peroxidase conjugated goat anti rabbit immunoglobulin (Miles). A further washing step was performed and the plates developed by incubation for 20 minutes with 100ul of substrate solution at room temperature. The reaction was stopped with 50ul of 2.5M H<sub>2</sub>SO<sub>4</sub> and the plates read with a Tietertek Multiskan spectrophotometer at 492nm.

#### 4.6.4 Results

##### a) Method of calculation

The optical densities for each serum sample tested were averaged and converted to ug/ml with a linear regression using least squares fit; this programme was developed by the Department of Medical Physics, Bristol Radiotherapy Centre, and gave a correlation coefficient of between 0.87 and 0.94.

This method proved simpler than reading the log concentration of the test sample from a graph of optical densities of the standard dilutions of P.L.A.P. and then converting to ug/ml.

##### b) Normal range

Serum levels in the normal population varied between 0.17ug/ml and 149.44ug/ml (mean 8.76ug/ml) in non smokers and 0.15 to 388.44ug/ml (mean 17.25ug/ml) in cigarette smokers. There was again a tendency overall for cigarette smokers to have higher levels of the NDOG2 determinant (Table 4.5).



When correlated with the number of cigarettes smoked:

1-10	18	range 0.15-23.29 ug/ml (mean 3.90ug/ml)
11-20	16	range 0.17-82.29 ug/ml (mean 6.51ug/ml)
21+	9	range 0.15-388.44ug/ml (mean 24.24ug/ml)

The possibility exists that endogenous peroxidase activity, from lysed red blood cells, may have given rise to these elevated levels in the normal population. However, the active sites on the plates should have been blocked with B.S.A. and any free peroxidase removed by washing. To test this hypothesis high normal sera were run in the assay but the step involving incubation with peroxidase conjugated goat anti rabbit immunoglobulin was omitted. No significant colour change was noted in the wells incubated with these samples demonstrating that endogenous peroxidase activity was not a source of error.

c) Serum half life of the NDOG2 determinant

The same series of sera, in the same patient as in section 4.4.5 (c), was studied using this assay and the results plotted on fig 4.3. Serum levels of 19.5ug/ml fell to 13ug/ml after 6 days and thereafter varied between 12 and 13ug/ml. There was therefore a fall of 6.5ug/ml in 6 days giving a similar half life to assay 1 (3-4 days).

d) Benign ovarian tumours

Levels of the NDOG2 determinant were measured in the same six patients studied in section 4.4.5 (d). Levels of the NDOG2 determinant were low ranging between 0.44 and 0.09 ug/ml (mean 0.198ug/ml) (Table 4.1).

e) Ovarian Cancer

The same three questions were asked of this assay as were asked of assay 1:

i) Do preoperative levels of the NDOG2 determinant correspond with the degree of reactive staining with NDOG2.

A very similar pattern to that observed with assay 1 was found in that those tumours with the larger proportion of their cells showing positive staining tended to have higher serum levels (Table 4.2).

Thus, the range for those tumours with Grade 3 and 4 reactive staining was 0.35-368.22ug/ml (Mean 68.19ug/ml).

Grade 2 reactive staining was 0.14-19.5ug/ml (mean 5.11ug/ml)

Grade 1 reactive staining was 0.1-2.81ug/ml (mean 0.65ug/ml)

ii) Does the NDOG2 determinant, as measured by this assay, act as a good tumour marker in ovarian cancer?

The same serum samples were run in this assay as in assay 1 and the results summarised in Tables 4.3 and 4.4.

Although the mean normal levels for non smokers was 8.76ug/ml, wide individual variations in levels were noted (0.17ug/ml-149.44ug/ml) with this assay. The significance of a single result was consequently difficult to interpret and so each patient was assessed on an individual basis; rising levels should correspond to

tumour recurrence and falling levels correspond to a response to therapy. Using these criteria, this assay accurately predicted the course of the disease in 20 patients. In 6 of these (patients 13, 20, 24, 38, 39 and 40) serum levels fell after treatment and did not subsequently rise (e.g. fig 4.4). These patients all remain well and are in remission. In 14 cases (patients 13, 6, 8, 12, 14, 21, 25, 30, 31, 32, 35, 36, 43 and 44) levels fell after surgery and initial chemotherapy but then subsequently rose reflecting recurrent tumour (e.g. fig 4.5). In two of these cases (patients 6 and 32) recurrent tumour had not been predicted by assay 1 (fig 4.6).

In a further 2 cases (patients 10 and 40) levels fell after surgery and chemotherapy but there was an unexplained single elevated level when the patients were in remission.

Subsequent serum levels were low and the patients remain well. Tissue was available for immunohistological examination in 16 of these 22 cases and showed grade 2 or greater reactive staining in 11 cases, grade 1 staining in 2 cases and was negative in 3 cases.

In 11 cases levels remained constant throughout the disease but did vary at some stage in the remaining 11. Three patterns of change were noted:

a) Levels only rose in the presence of massive recurrent disease in two cases (patient 1 and 34). Tumour tissue was only available from patient 34 and showed grade 2 reactive staining with NDOG2.

b) An inappropriate fall in serum levels were noted in 7 cases (patients 5, 16, 19, 22, 23, 37 and 41). In all of these cases serum levels had originally risen reflecting recurrent disease but then fell even though the tumour continued to grow.

c) False positive results were seen in the same 2 cases (patients 18 and 45) (fig 4.8) as in assay 1, and have been discussed earlier.

iii) Does the degree of reactive staining of the tumour with NDOG2 act as a guide as to whom should be followed up using this assay?

There were 15 patients whose tumours showed grade 2 or greater reactive staining with NDOG2 and this assay accurately predicted the course of the disease in 11 patients i.e. 73% (this figure includes patients 10 and 40 discussed in section 4.6(ii)). This assay, in common with assay 1, failed to predict the course of the disease in 4 patients. One of these (patient 18) was a cigarette smoker and this gave rise to a false positive result and another (patient 2) had a tumour of borderline malignancy whose pre and post operative and follow up serum levels did not vary. Both of these patients remain well.

In the case of patients 19 and 34, whose tumours showed grade 4 and grade 2 reactive staining respectively, this assay was not particularly useful. Patient 19's serum levels initially reflected the progress of the disease but then fell inappropriately shortly before her death

Serum levels only rose in patient 34, in the presence of gross disease. These findings may reflect a changing cell population within the tumour.

There were 12 patients whose tumours showed less than grade 2 reactive staining with NDOG2 and this assay predicted the course of the disease in 41% of these. 2 cases (patients 3 and 8) showed grade 1 and 3 cases (patients 21, 30 and 36) showed no reactive staining.

Thus, immunohistology does act as a guide as to who should be followed up but there are exceptions. In general the greater the degree of reactive staining, then the better the predictive value of this assay. This can be further improved if cigarette smokers and patients with tumours of borderline malignancy are excluded.

4.7 Comparison of results obtained with assays 1 and 3  
Overall, assay 3 accurately predicted the course of the disease in 50% of all the patients studied whereas assay 1 was accurate in 27%. The predictive values of both assays were improved if the tumour showed grade 2 or greater reactive staining with NDOG2 (60% assay 1, 73% assay 3).

The results with both assays were identical in 27 cases. In 11 cases both assays accurately reflected the course of the disease and in a further 12 cases there was no change throughout the disease with either assay. In both assays there were 2 false positive results and in one case levels only rose with bulk disease; in a further case levels fell inappropriately (Tables 4.3 & 4.4).

In 7 cases, assay 3 predicted the course of the disease when assay 1 either failed to do so (patients 3, 6, 8, 21, 30, 31 and 32) or fell inappropriately (patients 12, 15 and 44). In the case of patients 6 and 32 both assays showed a fall after primary surgery but assay 1 failed to show a rise in the presence of recurrent tumour (fig 4.6). Assay 3 was of some value in a further 4 cases (patients 5, 16, 37 and 41) in that it initially predicted the course of the disease but subsequently fell shortly before the patient's death; in these cases assay 1 was of no predictive value at any stage of the disease. In patient 1, assay 3 alone showed a rise but only in the presence of massive tumour recurrence.

There were 5 cases in which both assays initially agreed with each other in predicting the course of the disease but then subsequently one showed an inappropriate fall. In 3 of these (patients 12, 15 and 44) assay 3 was the more accurate and in all 3 the fall in assay 1 was mirrored initially by a lesser fall in assay 3. This subsequently rose but assay 1 showed continued low levels (e.g. fig 4.7). A change in chemotherapy correlated with this fall in one case only (patient 44) and a change back to the original regime did not result in a subsequent rise in assay 1.

In 2 cases (patients 22 and 23) an inappropriate fall was seen in assay 3 whereas a continued rise was seen with assay 1, but at a slower rate (e.g. fig 4.11). The fall in assay 3 corresponded to change in chemotherapy in patient 23.



#### 4.8 Affinity Chromatography using NDOG2 - sepharose, gel electrophoresis and immunoblotting

##### 4.8.1 Introduction

It became clear, from the previous sections that either assay 3 was a far more sensitive assay than assay 1 or that NDOG2 was capable of recognising at least one other tumour associated antigen. This latter possibility seemed the more likely in view of the very large range of normal levels and the different patterns seen in patients with ovarian cancer and in particular, cases 12, 15, 22, 23 and 44, where a fall in either assay resulted in a temporary drop or a slowing of the rate of increase of the other assay.

In this section 2 methods of assessing what, if anything, NDOG2 was recognising, in addition to P.L.A.P. in both normal and malignancy serum, were employed. The first method involved affinity chromatography with an NDOG2 - sepharose column and gel electrophoresis, while the second used an immunoblotting technique.

##### 4.8.2 Cyanogen Bromide activation of Sepharose and linking of the NDOG2 antibody

The object of this procedure is to covalently link the NDOG2 antibody to sepharose CL4B (Sigma) and thus to form a column for use in affinity chromatography.

### Method

Both Sepharose CL4B and the purified NDOG2 antibody need to be present in an activated form before covalent linkage can occur. The previously concentrated NDOG2 antibody is present in a P.B.S. buffer (3.5ug/ml) and this must be changed to a carbonate buffer pH 8.4 to activate NDOG2. This was done by gel filtration.

Sephadex G25 is swollen overnight in P.B.S. and 2, 12ml columns packed with this slurry. P.B.S. is used in preference to carbonate buffer (a) to clean the sephadex and (b) because the carbonate buffer pH may change overnight due to exposure to  $\text{CO}_2$  in the atmosphere. 12ml of a 0.1M carbonate ( $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ ) buffer pH 8.4 with 0.5ml NaCl (to stop electrostatic sticking) is run into each column. Further carbonate buffer was added to each column and the progress of the Dextran Blue observed. NDOG2 and Dextran Blue should travel through the column at the same rates, so when the Dextran Blue is at the bottom of its column the NDOG2 fraction will be ready to be collected. A further 4ml of buffer was run into the columns and the NDOG2 fraction was collected now present in carbonate buffer. A further 4ml of carbonate buffer is added to this fraction, bringing the total volume to 8ml.

Sepharose CL4B is supplied in a slurry with thiomersylene, preservative and is washed in distilled  $\text{H}_2\text{O}$ , in a sintered glass funnel connected to a Bruckner flask, to remove this. 12ml of Sepharose CL4B was added to 10ml of distilled

H<sub>2</sub>O in a beaker (this produced sufficient Sepharose for conjugation with 3 antibodies, of which NDOG2 was one) and the pH of this solution was made up to 11.0 with 1M NaOH. This beaker, with magnetic stirrer and a pH electrode attached was transferred to a fume cupboard prior to activation. 1gm of cyanogen bromide (Kodak) was weighed out in the fume cupboard and added to the Sepharose CL4B solution. The pH, which will tend to drop, is kept at 11.0 by the addition of further 1M NaOH. It is vital that the pH does not fall significantly as hydrogen cyanide gas will form at a pH of 8 or less. When the pH remained stable at 11 the reaction had ceased and this took 15 minutes from the time of adding the cyanogen bromide. All spatulae etc., that had come into contact with cyanogen bromide were washed in 1M NaOH.

The activated Sepharose was washed with 200ml H<sub>2</sub>O followed by 200ml carbonate buffer in the sintered glass funnel, in the fume cupboard. This step should not take longer than 90 seconds to stop the sepharose beads cross linking to each other.

The activate sepharose was then added to the NDOG2 solution, sealed and mixed overnight at 4°C.

This method results in 80-90% of the protein linking to the sepharose beads, the amount of free protein can be estimated by measuring the solution's optical density at 280<sup>0</sup> A and 8.9mg of NDOG2 was coupled to the beads. Further potential binding sites on the sepharose beads were

blocked by incubation with 20ml of 1M diethanolamine HCl pH 8 for 2 hours at room temperature. The beads were washed with 200ml each of carbonate buffer, 0.1M sodium acetate, 0.5M NaCl glacial acetic acid pH 4.1, carbonate buffer and finally P.B.S.. The beads were suspended in 20ml P.B.S. with azide and stored at 4°C until use.

#### 4.8.3 Running the NDOG2 column

125ul of serum from ovarian cancer patients with high and normal P.L.A.P. levels on serum assay 1 and from the normal control were run on the NDOG2 columns. These were prepared using 6 1ml syringes with glass wool packing their tips and a 21 gauge needle attached to direct the filtrates into the appropriate receiver. 3 syringes were packed to the 0.4ml mark (i.e. 3 x the load for each column) with NDOG2-sepharose and the other 3 syringes with sepharose linked to a control IgG1 recognising an antigen on the Epstein-Barr virus, these latter acted as controls to identify any non-specific sticking of protein to sepharose. All columns were washed twice with P.B.S. and checked to ensure no air bubbles were trapped in the column.

The serum samples were centrifuged at 13,000 r.p.m. to remove any large molecules that may block the columns. 125ul of the appropriate test sample was loaded into each pair of columns (i.e. 1 NDOG2 and 1 control) and incubated for 1 hour at room temperature, a further 30ul of test sample was added to two separate occasions during this time to aid spread of the test sample throughout the

column. The columns were then washed with 5ml P.B.S. and eluted using 0.05M diethylamine (D.E.A.) pH 11.5. 400ul of D.E.A. were initially run into the column and the pH of the filtrate measured, when this became alkaline a further 500ul of D.E.A. was run in and the filtrate collected. 125ul (i.e. 25% of the filtrate volume) Tris pH 8.0 was added to the filtrate to bring its pH nearer the physiological range.

#### 4.8.4 Polyacrylamide gel electrophoresis

##### a) Preparation

The protein in the filtrate must be resuspended in a running buffer before electrophoresis can be performed. Protein in the filtrate sample was precipitated by adding an equal volume (i.e. 625ul) of 15% trichloroacetic acid (T.C.A.) and incubating for 30 minutes at 4°C. T.C.A. has a minimum threshold for precipitating proteins and it is usual to add 1ug/ml of bovine serum albumin (B.S.A.) to the sample; however, as B.S.A. has a similar molecular weight to the subunits of P.L.A.P. this was not performed. T.C.A. will destroy the biological activity of proteins but this is not necessary for successful electrophoresis. After incubation the sample was centrifuged at 13000 r.p.m. the supernatant aspirated and the pellet resuspended in 500ul acetone and again centrifuged. This process was repeated, the supernatant aspirated and the pellet centrifuged to dry it.

The glass electrophoresis plates were washed and then cleaned twice with alcohol, followed by distilled water to remove any grease or protein from their surfaces. Spacers were placed between the plates and the edges sealed with vaseline. Bulldog clips held the whole together. 60ml of a 10% polyacrylamide separating gel consisting of:

- 1) 20ml acrylamide solution (30gm acrylamide and 0.8gm methylene bisacrylamide in 100ml);
- 2) 15ml of 1.5M Tris HCl pH 8.8;
- 3) 0.6ml of 10% S.D.S.;
- 4) 24.1ml  $H_2O$ ;
- 5) 0.02ml Temed;
- 6) 0.3ml 10% ammonium persulphate (A.P.S.) - which should be added last as this starts the reaction.

was poured between the plates to a line 1cm below the introducer.  $H_2O$  was added above this to stop the formation of a meniscus.

A 3% stacking gel was prepared consisting of:

- 1) 1.5ml of acrylamide solution;
- 2) 3.75ml of 0.5M Tris HCl pH 6.8;
- 3) 0.15ml of 10% S.D.S.;
- 4) 9.45ml  $H_2O$ ;
- 5) 0.01ml Temed;
- 6) 0.15ml 10% A.P.S..



H<sub>2</sub>O was emptied from above the set separating gel and the stacking gel was poured in. The introducer comb was added to form the sample wells.

The pellet was resuspended in 80ul of a gel sample buffer (0.5M Tris HCl, 10% S.D.S., glycerol, H<sub>2</sub>O with 1% B mercaptoethanol (M.C.E.), 0.5% bromphenol blue in a ratio of 1:1:1:6:8:0.2 and boiled for 5 minutes. M.C.E. will split P.L.A.P. into its subunits by breaking disulphide bridges.

b) Running

The bottom spacer was removed and the plate put into the electrode holder. Vaseline was again used as a seal. Electrode buffer, 3gm Tris, 14.4gm glycine and 10ml 10% S.D.S. in 1L H<sub>2</sub>O was added to the bottom well and all bubbles removed from under the gel. The introducer comb was removed after the bottom of the wells had been marked on the plate and the upper electrode well filled. 20ul of a mixture of proteins with known molecular weight were added to the wells at each end of the gel and the samples loaded into the remaining wells ensuring that they do not overflow.

A Volkam power pack, which gives a constant current was attached, with the positive lead to the bottom well and negative to the top well. The gel was run at a constant 40 M.A., although the voltage varied, for 4½ hours and all bubbles that formed were removed.

c) Staining and destaining

The gel was removed from the plates, the left side marked and the gel stained with 0.1% coomassie brilliant blue in methanol/acetic acid/water (5:1:4 by volume) overnight at room temperature. Destaining using methanol/acetic acid/water (1:1:8 by volume) took 48 hours at room temperature during which time the destaining solution was changed 3 times.

d) Results

No conclusive result was obtained by affinity chromatography due to a large amount of serum protein sticking, non-specifically to sepharose and showing on both the NDOG2 and control eluates. Albumin was present in relatively large amounts in both columns and, because of its molecular weight being very similar to that of the subunits of P.L.A.P., masked any P.L.A.P. present. Therefore an immunoblotting technique was next employed.

4.8.5 Immunoblotting

a) Introduction

Polyacrylamide gel electrophoresis is a convenient method of separating proteins but is difficult to handle and will not allow immunostaining of protein. Nitrocellulose membrane however, is convenient to handle and will allow immunostaining but is not used in electrophoresis.

The method of immunoblotting described here ("Western blot") involves the separation of proteins by polyacrylamide gel electrophoresis and their transfer to nitrocellulose membrane using a transblot electrophoretic transfer cell. The presence of the NDOG2 determinant can then be recognised by incubating with NDOG2 and then an enzyme linked second layer antibody followed by a suitable substrate.

b) Experiment 1

Method

Electrophoresis was performed using a 10% polyacrylamide gel to separate serum proteins from:

- 1) An apparently healthy individual with high levels of the NDOG2 determinant as measured by assay 3;
- 2) Patient 6. A preoperative sample of serum and a sample of serum taken at the time of tumour recurrence were tested. This patient had elevated levels in both assays preoperatively but only assay 3 predicted her tumour recurrence;
- 3) Patient 52. A preoperative sample of serum was tested. This patient's serum levels remained constantly normal in both assays;
- 4) P.L.A.P. (Sigma) at a concentration of 1mg/ml. The first well contained the standard marker protein suspension and the next 5 wells contained 10ul of the test samples boiled in 40ul G.S.B. which were repeated in the next 5 wells.

2 pieces of 3mm blotting paper and 1 piece of nitrocellulose were cut to the size of the polyacrylamide gel (16cm x 17.5cm) and soaked with 2 pads of "scotchbrite" in electroblot buffer (36g Tris, 172.6g Glycine, 1200ml methanol made up 6 litres). After electrophoresis, the gel was rinsed in electroblot buffer for 5 minutes and

the transblot cassette loaded, from anode to cathode, with "scotchbrite", paper, nitrocellulose, gel, paper, "scotchbrite" taking care to exclude all air bubbles. The cassette was closed and transferred to the transblot cell, containing electroblot buffer and run at 500 MA for 3 hours. Ice was packed around the outside of the cell to prevent overheating and a magnetic stirrer ensured that the buffer was kept circulating, to minimise any pH change due to electrolysis.

Prior to blotting the gel was measured so that, after the blotting step had been completed, the nitrocellulose membrane could be cut into 2 strips. The left half contained the marker proteins and the 5 test samples and the right half the 5 samples alone. The right half of the nitrocellulose membrane was transferred to a solution of 1% B.S.A., 1% haemoglobin in P.B.S., 0.02% azide and incubated overnight at 4°C. The lefthalf was stained for 10 minutes using an 0.5% solution of amino black in fix solution (500ml H<sub>2</sub>O, 450ml methanol, 50ml acetic acid) and then destained in fix solution to demonstrate the separation of the test proteins. The polyacrylamide gel was stained with coomassie blue as previously described (section 4.8.4 (c)) to confirm transfer of proteins from the gel to nitrocellulose.

The right hand half of the nitrocellulose membrane was washed in P.B.S., 0.05% Tween 20 after the blocking step and incubated with 15ml of NDOG2 supernatant in a plastic bag, for 1 hour at 4°C. The membrane was then washed

three times with P.B.S., 0.05% Tween 20 and incubated for 30 minutes with 15ml of a 1/150 dilution of an alkaline phosphatase conjugated sheep antimouse immunoglobulin (Sigma) in P.B.S., 0.5% B.S.A., 2% Hepes, at 4°C. A further four washes were performed with P.B.S., 0.05% Tween 20 followed by one wash with T.B.S. pH 7 buffer.

Fresh substrate consisting of 15mgm of Fast Red T.R. salt (Sigma) in 15ml of a solution containing 0.3mg Napthol ASMX dissolved in 0.3ml diethyl formamide in 14.7ml 0.1M Tris HCl pH 8.2, filtered using Whatmans No. 3 filter paper, was prepared and incubated with the nitrocellulose membrane for 30 minutes, at room temperature, in a fume cabinet. The membrane was then washed with T.B.S. and any positive reactive staining showed as a red band.

### Results

10ul of 'neat' test serum per well resulted in overloading of both the gel and nitrocellulose paper with serum proteins. The strip stained with amido black confirmed that protein had been transferred but no meaningful result was obtained from the strip incubated with NDOG2.

### c) Experiment 2

#### Method

Experiment 1 was repeated but in this case 10ul per well of a 1 in 200 dilution of each test serum was run on 2 polyacrylamide gels. The blotting procedure was repeated but the 2 cassettes used required 1.1 amps for 3 hours to transfer protein from the gel to the nitrocellulose paper.

4 strips of paper were therefore obtained each containing 1 row of sigma P.L.A.P. and the 4 test sera. The first strip was stained with amido black, the second was incubated with NDOG2 and then with an alkaline phosphatase conjugated sheep anti mouse immunoglobulin. The third strip was incubated with P.B.S./0.05% Tween 20 and acted as a control; the fourth strip was incubated first with the rabbit anti human P.L.A.P. immunoglobulin (Dako) used in assay 3 and then with an alkaline phosphatase conjugated goat anti sheep immunoglobulin. The colour was developed as in experiment 1.

### Results

The paper was not overloaded in this case, and both NDOG2 and the rabbit anti human P.L.A.P. antiserum recognised a band at 67,000 Daltons in the sigma preparation. In addition the polyclonal preparation recognised a number of bands of lower molecular weight in this preparation. These may have been due to P.L.A.P. degradation products, not recognised by NDOG2 or the presence of contaminants in the sigma preparation recognised by the polyclonal antibody alone.

The strip incubated with the polyclonal preparation also showed a very faint band at 67,000 Daltons in the positive (patient 6) recurrent (patient 6) and high 'normal' serum samples.

These were not detected by NDOG2, possibly due to the low concentrations of P.L.A.P. present in 10ul of a 1 in 200 dilution of test serum. Polyclonal antisera detect a



number of antigenic sites on any one protein, whereas a monoclonal will detect only one. Therefore, many more molecules of the rabbit antihuman P.L.A.P. antiserum will attach to the P.L.A.P. molecule than NDOG2. This may explain why although both recognise the sigma P.L.A.P. preparation (a high concentration), only the strip incubated with the polyclonal antiserum showed a band at 67,000 Daltons in the high and recurrent serum samples.

### Experiment 3

#### Method

600ul of test serum from patients with:

- a) High serum levels in both assays;
- b) Low serum levels in both assays;
- c) A 'normal' case with normal levels in assay 1 but high levels in assay 3.

and 600ul of a preparation containing 200ug of P.L.A.P. (sigma) in P.B.S., were all mixed for 2 hours at 4°C with 400ul of NDOG2-sepharose. The beads were separated by centrifugation at 10,000 r.p.m. for 5 minutes and then washed with 1ml P.B.S., three times.

The beads were transferred to a fresh tube, washed again and then boiled with 400ul of gel sample buffer for 5 minutes. The beads were separated by centrifuging at 1000 r.p.m. for 5 minutes and the supernatant saved.

2 10% polyacrylamide gels were prepared and loaded with 80ul/well of each sample as follows:

WELL ( 1 (	2 7	3 8	4 9	5 10	6 11	12
Markers	PLAP	PLAP	Sample a	b	c	Blank
		AFTER INCUBATION WITH NDOG2- SEPHAROSE				

Experiment 2 was repeated but used purified NDOG2 in a concentration of 10ug/ml.

### Results

i) The strip of nitrocellulose stained with amido black showed the separation and transfer of proteins from the test samples;

ii) The control strip showed bands equivalent to the heavy and light chains of NDOG2 due to elution of the antibody from sepharose;

iii) The strip incubated with rabbit anti human P.L.A.P. immunoglobulin showed a band at 67,000 Daltons, the same molecular weight as P.L.A.P. subunits, in the sigma P.L.A.P. sample faintly in test samples (a) and (c) and in the sample of P.L.A.P. previously incubated with NDOG2-sepharose. This latter showed fewer of the lower molecular weight bands previously demonstrated in experiment 2 (4.8.5 (c)). As the 67,000 Dalton band was still visualised in samples (a) and (c) after incubation with NDOG2-sepharose it is unlikely that this was due to intestinal alkaline phosphatase.

iv) The strip incubated with NDOG2 showed bands equivalent to the heavy and light chain only. No bands were seen with the P.L.A.P. (Sigma) samples or test sera. This result may be due to the NDOG2-sepharose binding only a small amount of P.L.A.P., which, while sufficient to be detected by the polyclonal antibody, might still be too low to be visualised using NDOG2.

#### 4.9 Conclusions and discussions

This chapter described the development of 2 assays, based on NDOG2, that have been used to monitor patients with ovarian cancer. The first of these, assay 1, measured enzymically active P.L.A.P. and the second, assay 3, measured protein captured by NDOG2. There were many similarities between these 2 assays:

- 1) The half life of the antigen(s) recognised was the same in both assays;
- 2) Cigarette smoking was a cause of raised serum levels with both assays;
- 3) There was a close correlation between preoperative serum levels and the degree of reactive staining of the ovarian carcinoma in both assays;
- 4) Low preoperative serum levels were recorded with both assays in patients with benign ovarian tumours.
- 5) Both assays accurately predicted the course of the disease in the same 11 cases (section 4.7).

There were also several differences:

- 1) The most striking of these was the unexplained high levels recorded with assay 3 in some healthy blood donors;
- 2) In 7 cases (patients 3, 6, 8, 21, 30, 31 and 32) assay 3 accurately reflected the course of the disease when assay 1 did not;
- 3) In 5 cases (patients 12, 15, 22, 23 and 44) both assays initially agreed but then one showed an inappropriate fall in the presence of residual tumour. The other assay either briefly fell and then continued to rise (e.g. fig 4.7) or rose at a slower rate (e.g. fig 4.11). This latter case suggests a high initial concentration inactive protein as active enzyme could only rise whilst total protein (assay 3) fell under the circumstances.

These differences suggest that NDOG2 recognises active P.L.A.P. and a second tumour associated antigen. This second antigen has not been clearly identified but it is obviously very closely related to P.L.A.P.. It must have one epitope recognised by NDOG2 and at least one other recognised by the polyclonal rabbit anti human P.L.A.P. antiserum. This latter is known to cross react with human intestinal alkaline phosphatase (Dako Data Sheet). The proposed second antigen possibly has a molecular weight identical to that of an alkaline phosphatase subunit as a faint band at 67,000 Daltons was detected by the polyclonal antiserum (but not NDOG2) in the high normal serum sample (experiments 2 & 3, (4.8.5)). Incubation of higher volumes of test serum with NDOG2-sepharose

prior to blotting (experiment 1 (4.8.5)) still resulted in insufficient protein being transferred to the nitrocellulose paper to be visualised using NDOG2.

The second antigen may be an inactive form of P.L.A.P. or an inactive hybrid alkaline phosphatase molecule which would consist of one P.L.A.P. and one intestinal alkaline phosphatase subunit. However, it could also be a separate serum protein, with no phosphatase activity equivalent to the low molecular weight bands detected in the sigma P.L.A.P. preparation (experiments 2 & 3 (4.8.5)).

The predictive value of both assays was improved when only patients with positive immunohistology were followed up. Even so assay 1 was only accurate in 60% of these and assay 3 in 73%. This is possibly due to the lower expression of the NDOG2 determinant by secondary tumour deposits (Chapter 3).

Cigarette smoking is the major "benign" cause of elevated serum P.L.A.P. and can therefore cause confusion in the interpretation of both assays in such cases (e.g. fig 4.8).

4.10 Tables, Charts and Figures

Table 4.1 Preoperative serum levels in patients with benign ovarian tumours compared with degree of reactive staining with NDOG2

Patient	Histological Type	Degree of staining NDOG2	Assay 1	Assay 3
1	Fimbrial cyst	Grade 3	0.0 I.U./L	0.14 ug/ml
2	Serous Cystadenoma	Grade 1	0.21 I.U./L	0.09 ug/ml
3	Serous Cystadenoma	Grade 3	0.0 I.U./L	0.11 ug/ml
4	Serous Cystadenoma	Grade 2	0.0 I.U./L	0.2 ug/ml
5	Serous Cystadenoma	Grade 2	0.0 I.U./L	0.44 ug/ml
6	Serous Cystadenoma	Grade 3	0.0 I.U./L	0.21 ug/ml



Table 4.2 Comparison of the degree of reactive staining with NDOG2 of the primary tumour and preoperative serum levels using assay 1 and 3 in non smoking patients with ovarian cancer

Patient	Histology	Immuno-histology	Assay 1 (I.U./L)	Assay 3 (ug/ml)
6	Serous	Grade 4	5.6	0.35
13	Serous	Grade 4	5.6	36.02
24	Serous	Grade 4	2.55	3.35
32	Serous	Grade 4	0.45	0.84
2	Serous (borderline malignancy)	Grade 3	<0.1	0.37
38	Serous	Grade 3	8.5	368.22
20	Serous	Grade 2	0.95	19.5
33	Serous	Grade 3	2.1	0.2
35	Serous	Grade 2	1.5	0.6
43	Adenocarcinoma	Grade 2	<0.1	0.14
7	Adenocarcinoma	Grade 1	<0.1	0.16
8	Serous	Grade 1	<0.1	0.89
21	Adenocarcinoma	Negative	<0.1	2.81
30	Anaplastic	Negative	<0.1	1.51
36	Adenocarcinoma	Negative	0.35	0.3
37	Serous	Negative	<0.1	0.41
46	Adenocarcinoma	Negative	<0.1	0.13
50	Mucinous	Negative	<0.1	0.13
52	Adenocarcinoma	Negative	<0.1	0.1
4	Serous	Necrotic	<0.1	0.11

Table 4.3 Comparison of the immunohistology of the tumour with the predictive value of assays 1 and 3

Patient	Course of Disease	Histology	Degree of Reactive Staining	Predictive value?	
				Assay 1	Assay 3
6	R	S	Grade 4	N	Y
13	W	S	Grade 4	Y	Y
19	D	S	Grade 4	(Y)	(Y)
24	W	S	Grade 4	Y	Y
32	R	S	Grade 4	N	Y
2	W	S (B.M.)	Grade 3	N	N
10	W	S	Grade 3	Y	Y
18	W	S	Grade 3	F.P.	F.P.
38	W	S	Grade 3	Y	Y
39	W	S	Grade 3	Y	Y
20	W	S	Grade 2	Y	Y
34	D	A	Grade 2	B	B
35	R	A	Grade 2	Y	Y
50	W	S	Grade 2	Y	Y
43	R	A	Grade 2	Y	Y
7	W	A	Grade 1	N	N
8	R	S	Grade 1	N	Y
11	W	S	Grade 1	N	N
22	R	S	Grade 1	Y	(Y)
45	W	M	Grade 1	F.P.	F.P.
3	D	S	Grade 1	N	Y
36	D	A	Negative	Y	Y
21	R	A	Negative	N	Y
30	D	A	Negative	N	Y
37	D	S	Negative	N	(Y)
42	R	A	Negative	N	N
4	W	S	Necrotic	N	N

R = Recurrence

D = Death

W = Well

S = Serous cystadenoma

M = Mucinous cystadenoma

A = Adenocarcinoma

(B.M. = Borderline malignancy)

Y = accurately predicts

N = levels never elevated

B = levels only elevated with bulk disease

(Y) = levels fall inappropriately

F.P. = False positive

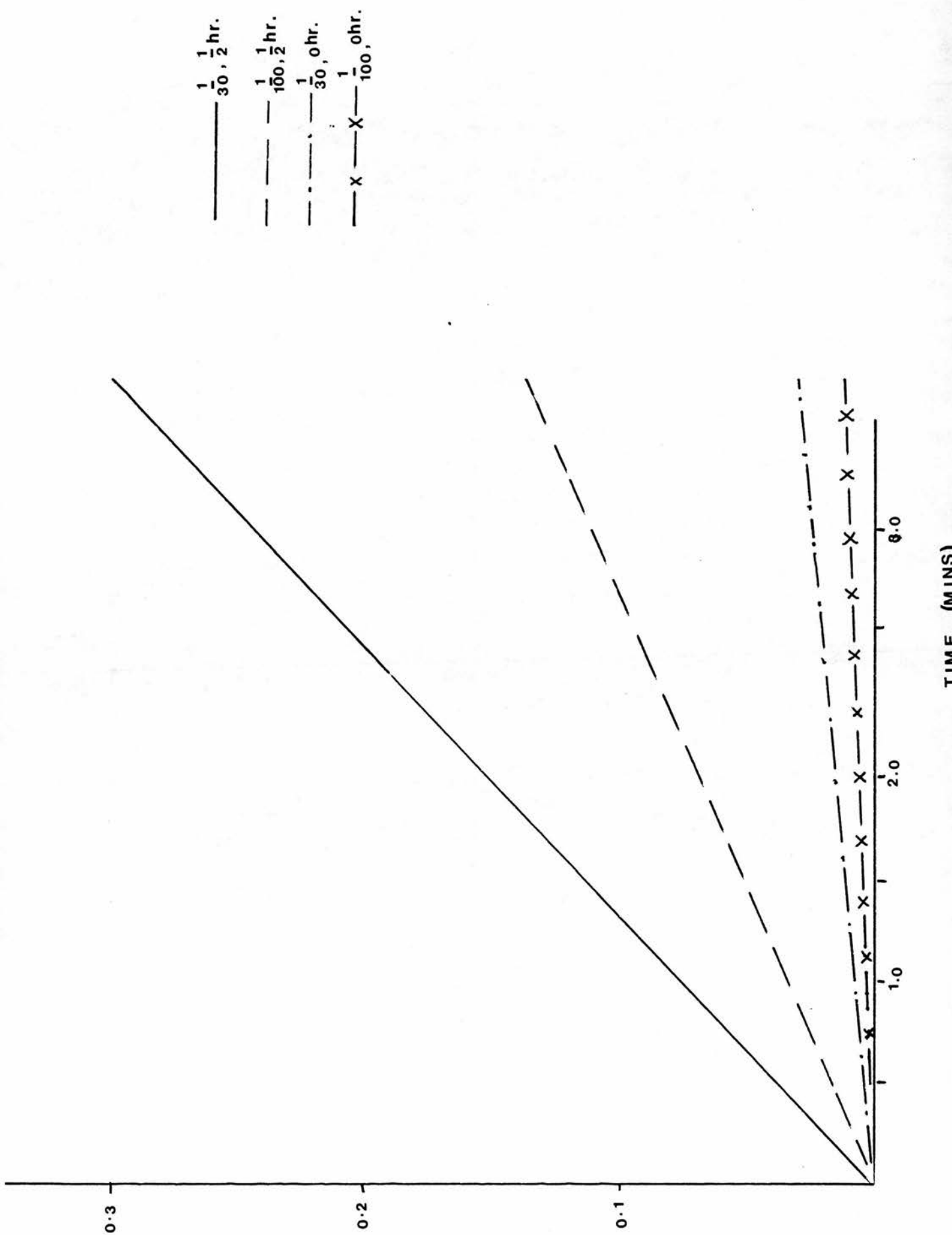
Table 4.4    The value of assays 1 and 3 in predicting the course of the disease in those patients in whom no immunohistology was performed

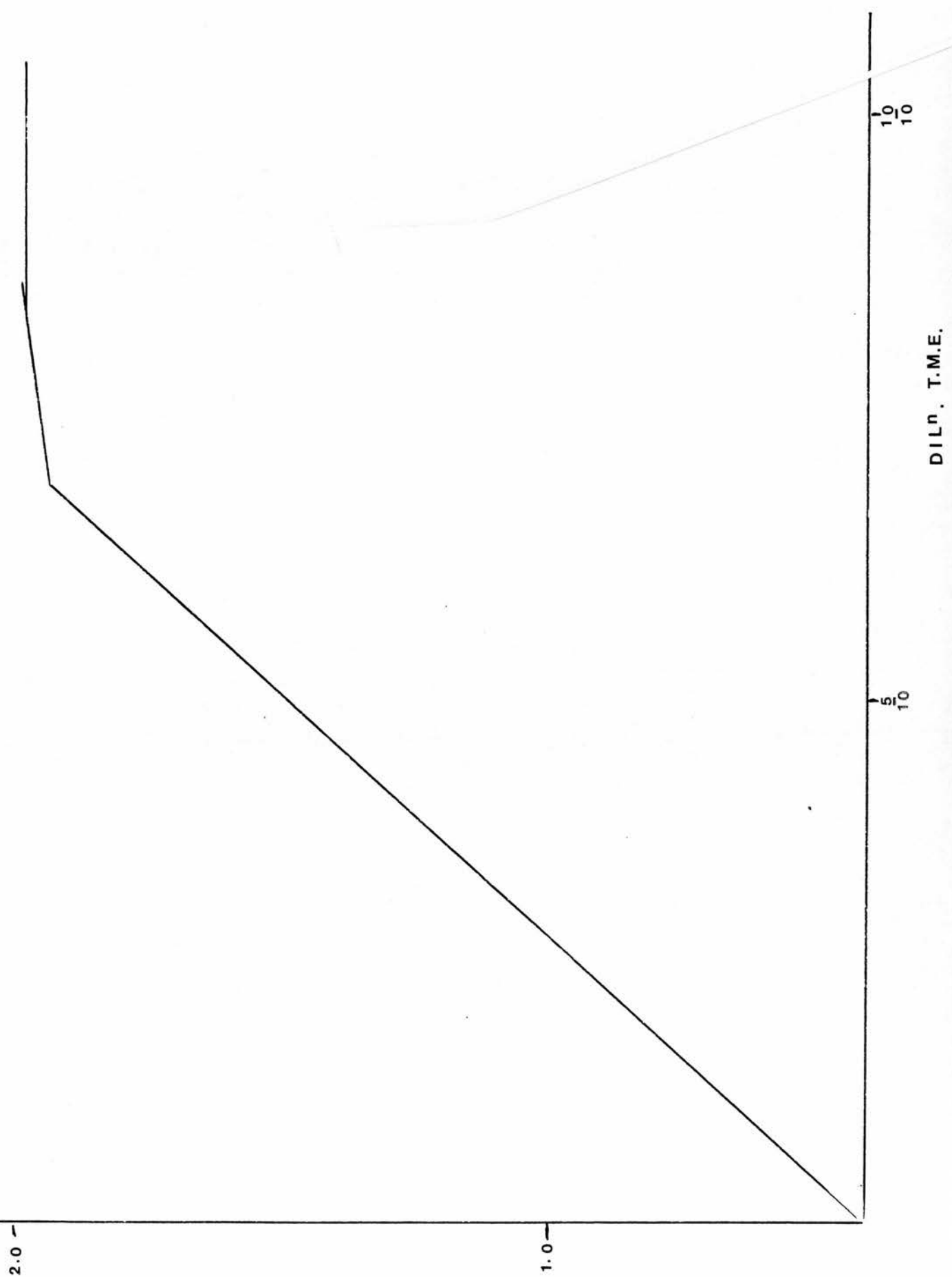
Patient	Course of the Disease	Histology	Predicts the course of the disease?	
			Assay 1	Assay 3
1	R	S	N	B
5	D	S	N	(Y)
16	D	M	N	(Y)
28	W	S	N	N
31	W	S	N	Y
41	D	S	N	(Y)
29	W	S	N	N
49	R	A	N	N
48	D	A	N	N
47	D	A	N	N
14	R	A	Y	N
23	D	A	Y	(Y)
12	D	S	(Y)	Y
15	R	A	(Y)	Y
25	R	S	Y	Y
44	D	A	(Y)	Y
17	R	A	N	N

Table 4.5 Variation in levels of P.L.A.P. determined by Assay 3 in the normal population

Level of P.L.A.P.	Number of patients			
	Non-smoking		Smoking	
<1.0 ug/ml	37/66	56%	23/44	52%
<1.5 ug/ml	45/66	68%	27/44	61%
<3.0 ug/ml	50/66	76%	29/44	65%
<4.0 ug/ml	51/66	77%	34/44	77%
<5.0 ug/ml	53/66	80%	36/44	82%
<6.0 ug/ml	54/66	82%	36/44	82%
<10.0 ug/ml	57/66	86%	39/44	89%
<15.0 ug/ml	59/66	89%	39/44	89%
<50.0 ug/ml	62/66	94%	41/44	93%
<100.0 ug/ml	64/66	97%	42/44	95%
<200.0 ug/ml	66/66	100%	43/44	98%
<300.0 ug/ml	66/66	100%	43/44	98%
<400.0 ug/ml	66/66	100%	44/44	100%

Demonstration of long term product inhibition of P.L.A.P.

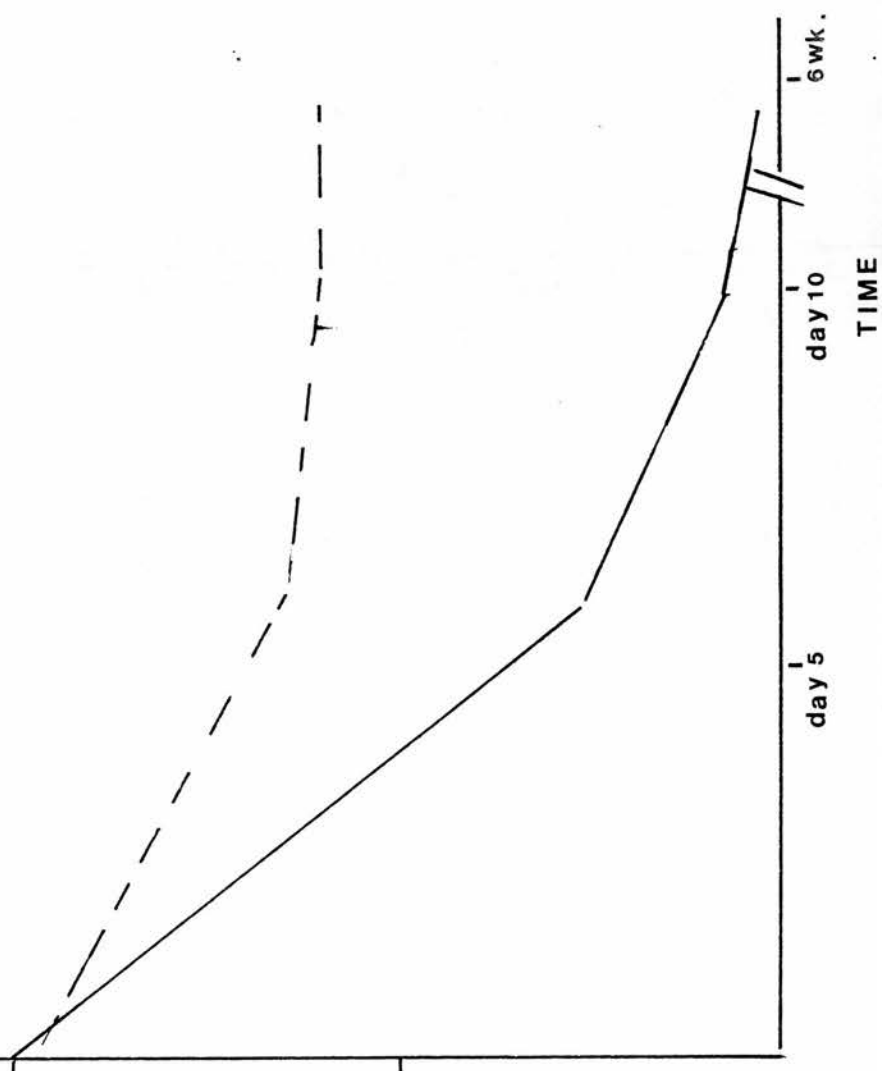




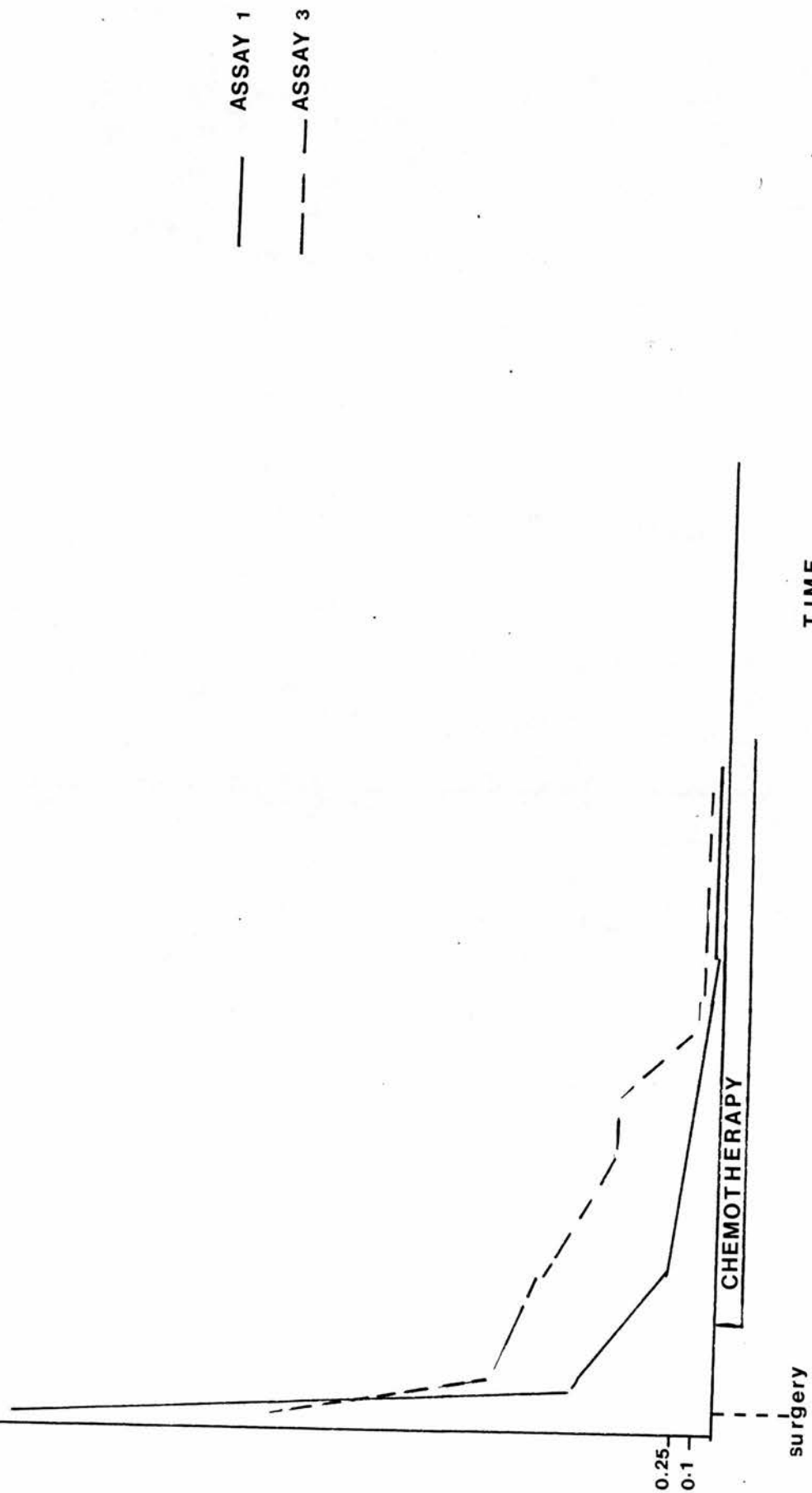


Half life of P.L.A.P. in serum measured by Assays 1 and 3

— ASSAY 1  
- - - ASSAY 3

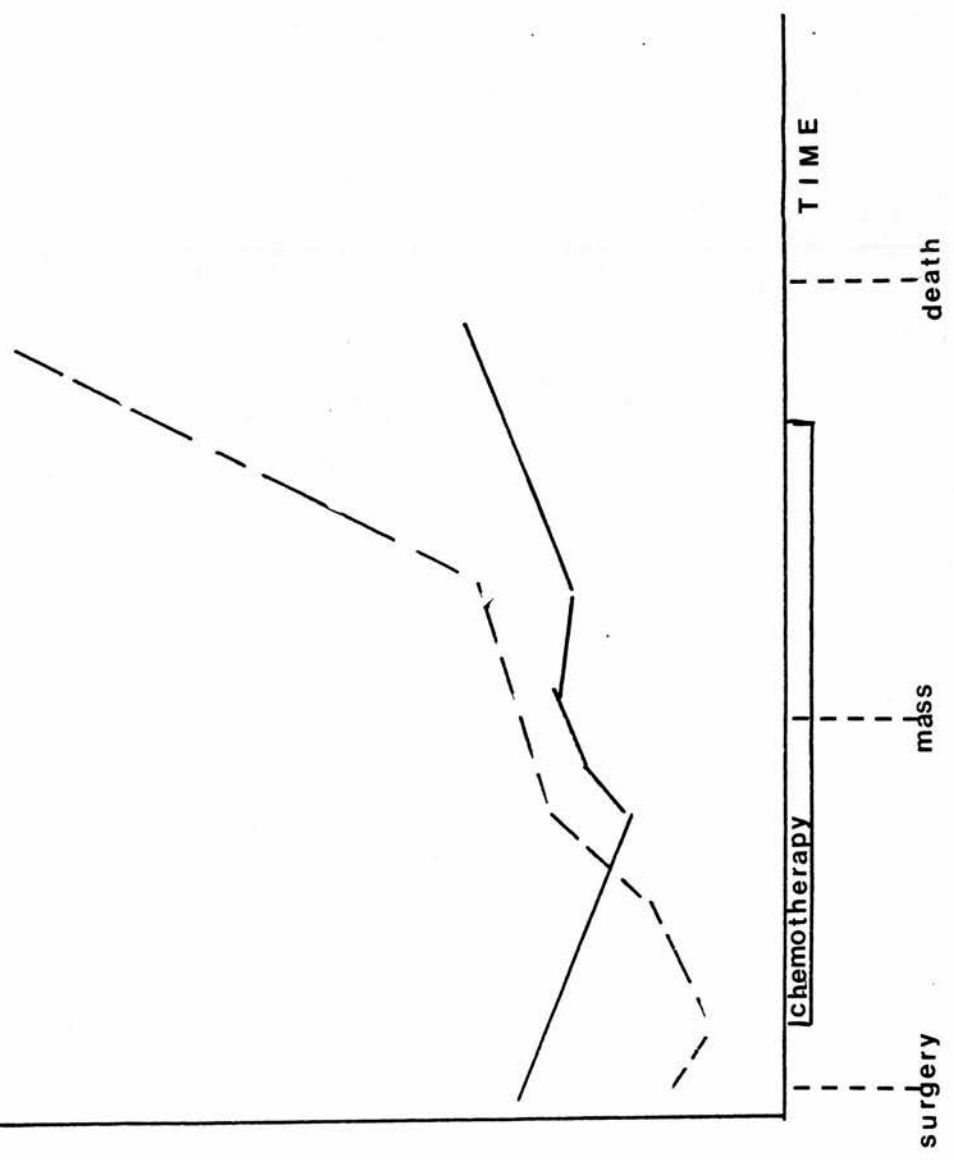


... who is a non smoker. Her moderately well differentiated serous cystadenocarcinoma was incompletely excised at operation but she responded well to chemotherapy.



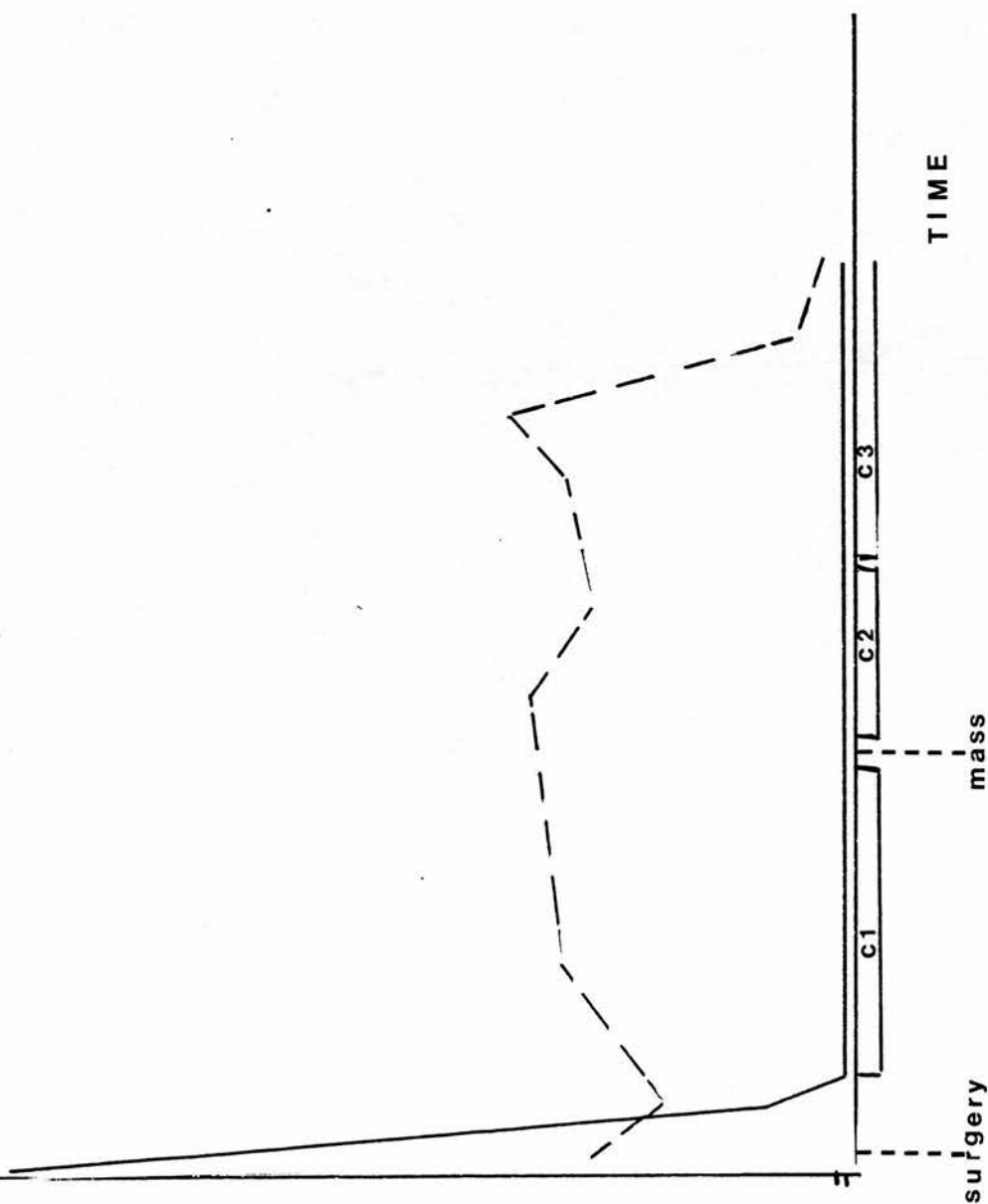
A 63 year old lady who had residual disease left after primary surgery. Despite chemotherapy she developed an abdomino-pelvic mass and eventually died of her disease. This patient was a non smoker.

— ASSAY 1  
- - - ASSAY 3

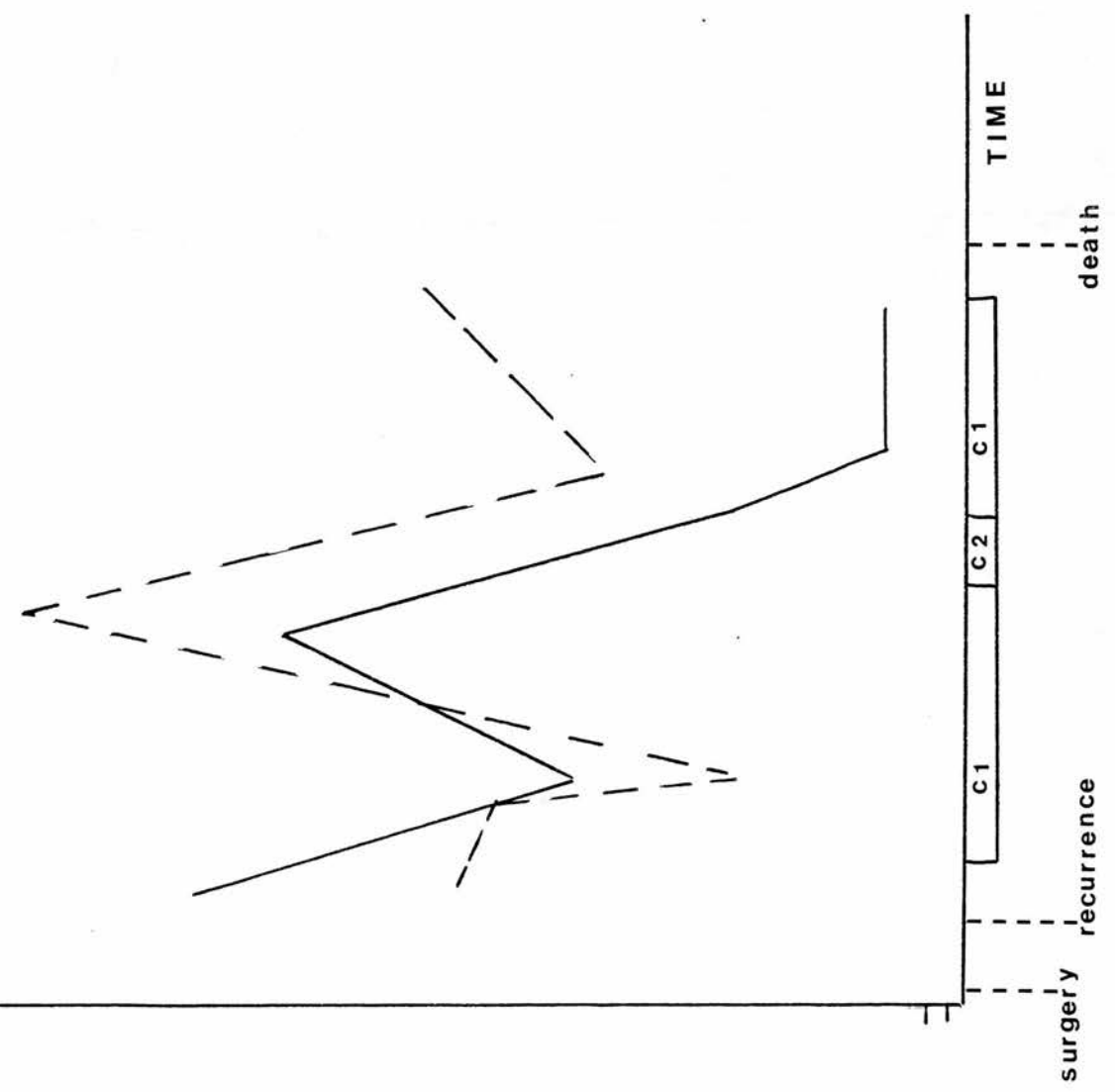


incompletely excised at surgery. This patient failed to respond to cisplatinum (C 1) or chlorambucil (C 2) but did respond to cyclophosphamide (C 3). Enzymically active P.L.A.P. poorly reflected the course of the disease but the NDOG2 determinant, as measured by assay 3, did.

— ASSAY 1  
- - - ASSAY 3



... year old lady with a stage IC anaplastic serous  
cystadenocarcinoma who developed a pelvic recurrence  
soon after surgery. This patient died of her disease  
despite chemotherapy. Both assays initially reflected  
the course of the disease but assay 1 fell inappropriately  
in the latter stages of the disease.



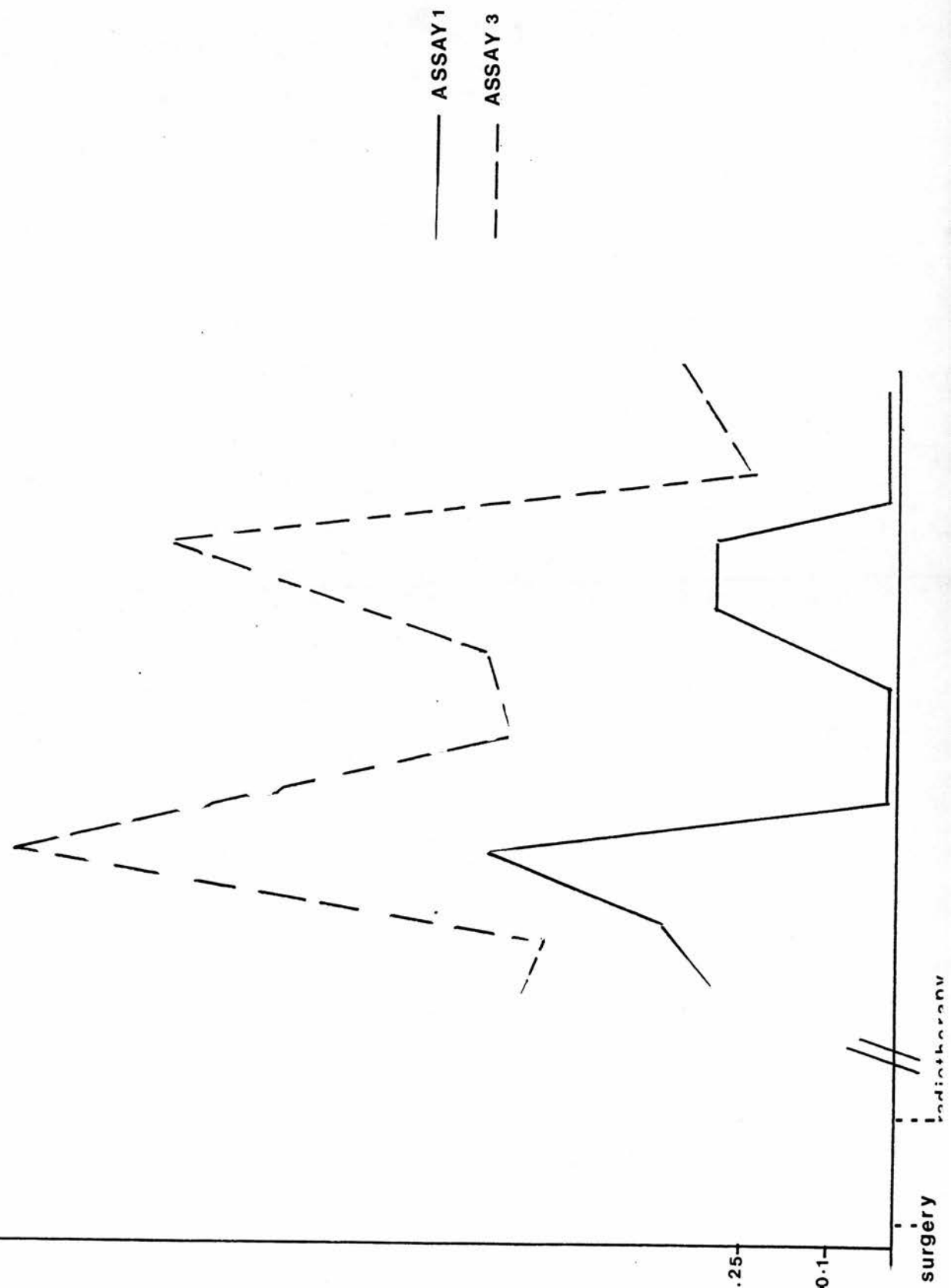
— ASSAY 1  
- - - ASSAY 3

C1=vinblastine, methotrexate, bleomycin  
C2=treosulphan

SERUM  
LEVELS

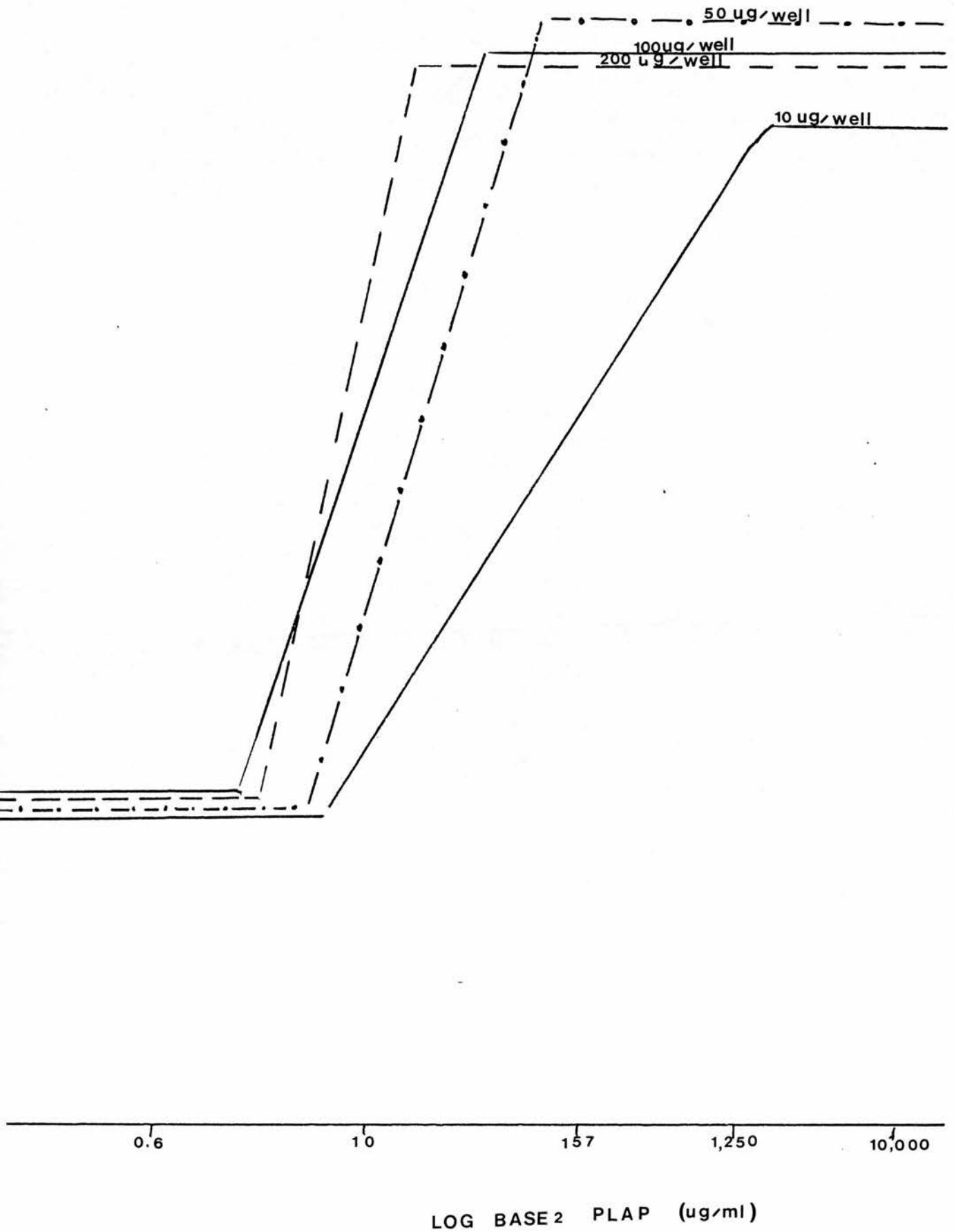
Fig 4.8 Patient 18

A 48 year old lady treated with surgery and radiotherapy for a stage Ia serous cystadenocarcinoma. The patient smoked on average 20 cigarettes a day and remains well despite elevated P.L.A.P. levels.

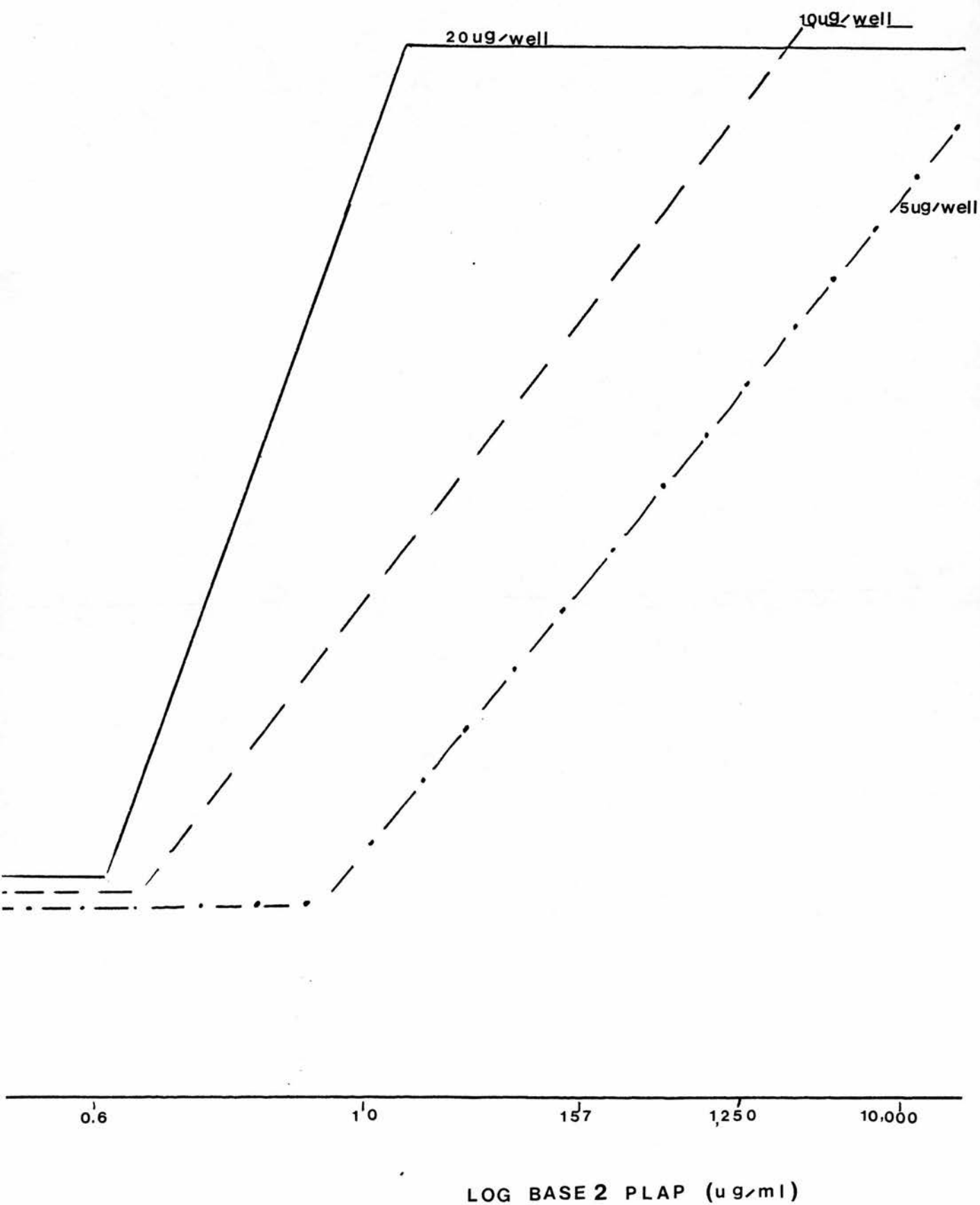




Titration curves. Assay 3 with coating concentrations of 200ug, 100ug, 50ug and 10ug NDOG2 per well.

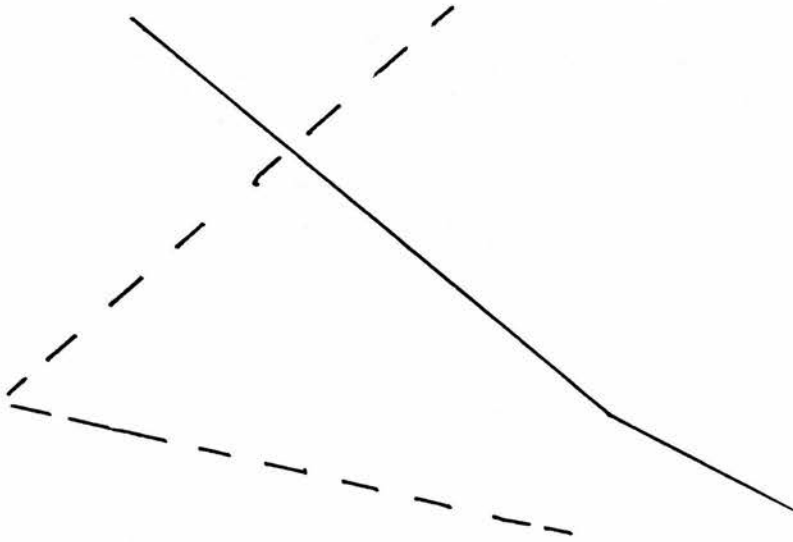


Titration curves Assay 3 with coating concentrations of 20ug, 10ug and 5ug NDOG2 per well



carcinoma, that did not respond to chemotherapy. Assay 1 more accurately reflected the course of the disease.

SERUM  
LEVELS



— ASSAY 1

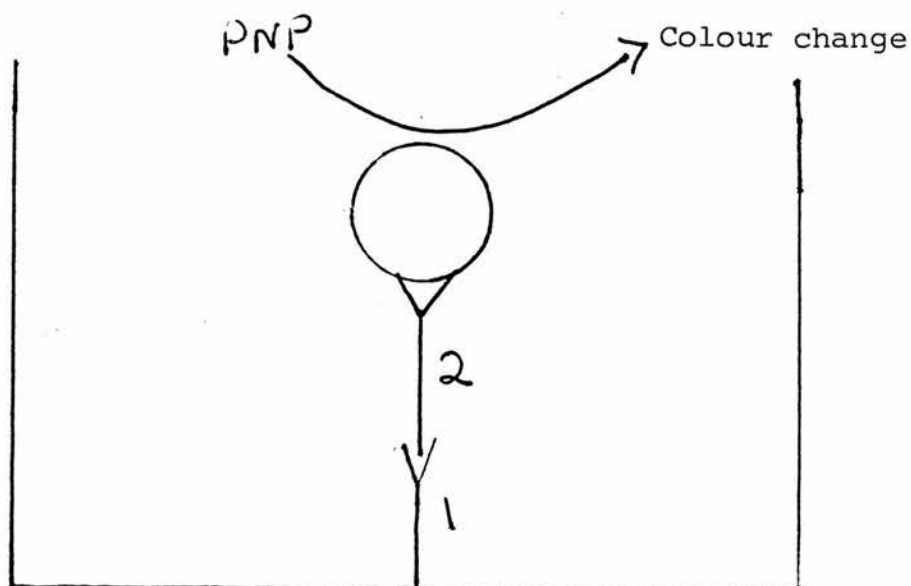
- - - ASSAY 3

c1 = cisplatin

c2 = treosulphan

c3 = chlorambucil





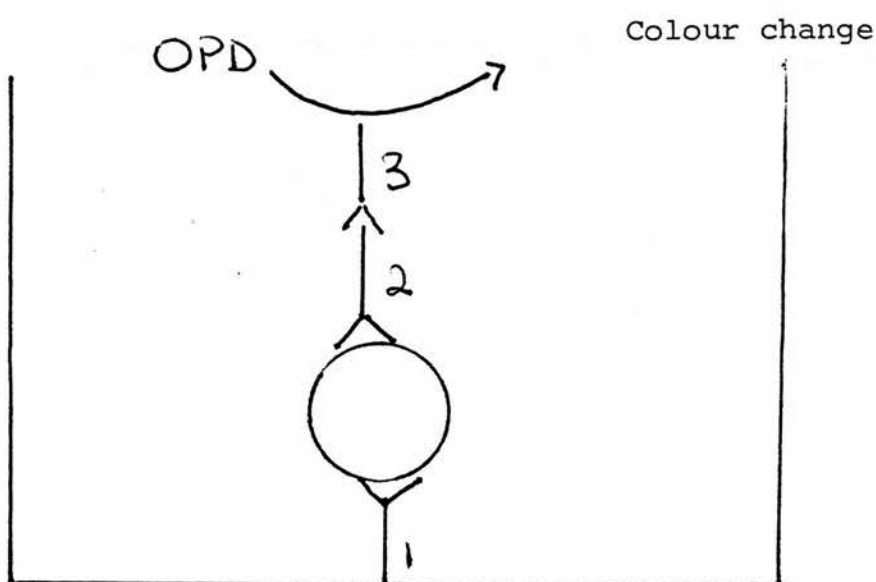
Y<sub>1</sub> = Rabbit anti mouse immunoglobulin

Y<sub>2</sub> = NDOG2

○ = P.L.A.P.

PNP = Paranitrophenol phosphate

Fig 4.13 Demonstration of Assay 3



Y<sub>1</sub> = NDOG2

Y<sub>2</sub> = Rabbit anti human P.L.A.P. immunoglobulin

Y<sub>3</sub> = Peroxidase conjugated goat anti rabbit antiserum

○ = P.L.A.P.

OPD = Orthophenyl diamine

## CHAPTER 5

RADIOIMMUNODETECTION OF TUMOUR USING  $^{123}\text{I}$ -IODINE

LABELLED NDOG2

### 5.1 Introduction

Ovarian cancer, with its propensity for intra-abdominal metastasis, presents certain problems in the detection of early tumour recurrence and in assessing a patient's response to therapy. Conventional imaging techniques such as ultrasound scanning (U.S.S.) and Xray computed tomography (C.T.) have limited value in the detection of small, solid intra-abdominal tumour deposits, particularly after surgery. Radioimmunoscintigraphy (R.I.S.) which is an imaging technique using radiolabelled antibodies, has shown promise in the detection of small intra-abdominal tumour deposits (Van Nagell et al 1978, Granowska et al 1984).

This chapter describes the use of <sup>123</sup>-iodine labelled NDOG2 antibody in R.I.S. of patients, primarily those with ovarian cancer but also those with breast, testicular and endometrial cancers. The NDOG2 determinant is present on the three common allelic forms of P.L.A.P. and has been demonstrated immunohistologically in over 60% of epithelial ovarian cancers and over 50% of endometrial carcinomas. Both testicular and breast tumours have been reported to express P.L.A.P. (Lange et al 1982, McDicken et al 1983). The strongest reactive staining with NDOG2 in normal tissues was seen in fallopian tube, endometrial and endocervical epithelium (Chapter 3), organs that, in the case of ovarian cancer, will usually have been removed at the time of primary surgery. Lung and thymus showed variable staining with NDOG2 and in the latter case staining was minimal and intracellular. Therefore, from the point of potential uptake by normal tissue NDOG2 compares favourably with other antibodies used in R.I.S. e.g. HMFG<sub>2</sub> (Epenetos et al 1982).



## 5.2 Methods

### 5.2.1 Patients

R.I.S. was performed in 27 patients, of these 20 had known or suspected ovarian cancer, 5 patients had metastatic breast cancer and 1 patient, each, was imaged with an endometrial carcinoma and a disseminated seminoma. 14 patients in the ovarian cancer group were imaged prior to or shortly after surgery, 5 patients were imaged after a course of, or prior to a change in, chemotherapy and the final patient was imaged after radiotherapy.

Informed consent was obtained in all cases and any patient with a known allergy to mice or iodine excluded from this study. All patients received a thyroid blocking agent. For the first 9 patients 400mg of potassium perchlorate was given orally 2 hours before imaging, followed by 200mg daily for the next 3 days. This proved to be only partly effective in blocking thyroid uptake of 123-iodine ( $^{123}\text{I}$ ) and so potassium iodide, in a dose of 420mg either 14 or 2 hours before imaging and 180mgm daily for 3 days after imaging was used. This was fully effective.

Patients in the ovarian cancer group were selected because of positive immunohistology of their tumour alone (6 patients) elevated serum P.L.A.P. (either assay) alone (4 patients) or both parameters (6 patients). 3 patients were imaged without prior knowledge of the P.L.A.P. status of their tumour and one patient was imaged whose tumour did not apparently produce P.L.A.P. (Table 5.1).

5 patients with breast carcinoma, in whom there was no evidence of abdominal or pelvic metastases were imaged. Breast cancer does express P.L.A.P. although serum levels may be normal (McDicken et al 1983); however the primary aim of imaging this group of patients was to obtain a series of scans of disease free pelvis and abdomens.

The patient with an endometrial carcinoma had both positive immunohistology and elevated serum P.L.A.P. levels whilst the patient with disseminated seminoma, who was also a heavy smoker, had elevated serum P.L.A.P.. No fresh tissue was available in this latter case.

#### 5.2.2 Purification of NDOG2

The NDOG2 antibody used in this chapter was produced by NDOG2 tumours growing in the peritoneal cavities of Balb/c mice. The ascites produced by these animals was collected and the antibody purified by Protein A sepharose affinity chromatography and sephacryl S300 column chromatography (section 4.3.6 (ab) and (b)). The antibody was concentrated to 2.6mg/ml (section 4.3.6. (d)), divided into 100ul (i.e. 260ug) aliquots and stored at  $-20^{\circ}\text{C}$  until use. This preparation gave no side effects when injected into 2 rats and 2 guinea pigs at a concentration of 70ug per animal.

#### 5.2.3 Choice of Radionuclide

The various isotopes used in R.I.S. have been discussed in Chapter 2.  $^{123}\text{I}$  was used in this study because it is a pure gamma emitter with an energy range ideally suited to the modern gamma camera and because simple and effective methods exist for the iodination of proteins. Although free iodine is taken up by the thyroid gland this can be prevented as described in 5.2.1..

#### 5.2.4 Method of Iodination

##### a) Theory

Two methods of iodinating antibodies for use in R.I.S. exist, the iodogen (Epenetos et al 1982) and the chloramine T methods. The latter, originally described by Hunter and Greenwood (1962), was used in this study.

Iodine will bind to the aromatic side chain of tyrosine but also, weakly to histidine and phenylalanine, whether they are free aminoacids or in proteins. Chloramine T, a potent oxidising agent, converts iodine to a more reactive form and enhances its binding capacity.

##### b) Method

The iodination of NDOG2 was performed in the 'sterile' room of the Radiopharmacy Department at the Bristol General Hospital. All reagents used were sterilised either by autoclaving at 115°C 15lb/square inch for 30 minutes (P.B.S. and Sephadex G50), heat sterilisation at 2000°C for 2 hours (glassware), ultrafiltration (P.B.S., sodium phosphate, saturated tyrosine and Chloramine T solutions, NDOG2 antibody) or supplied sterile by the manufacturers (Human plasma protein fraction (H.P.P.F.), Bijoux, syringes and needles).

The 10ml fractionating column was made from a glass pipette with the top removed and a cotton wool plug firmed down with the tip. Sephadex G50 was swollen overnight with excess P.B.S., which was subsequently decanted and a further 20-30ml P.B.S. added. The resultant mixture was pipetted into the column, without the inclusion of any air, to the

10ml mark. 1ml H.P.P.F. was added to block any non-specific protein binding to the column. The column, which has a capacity of 3ml, was washed with 3ml P.B.S. to remove free H.P.P.F. and kept topped up with P.B.S. to avoid any drying out of the column.

500mBq of dry sodium  $^{123}\text{I}$ -iodine (Harwell) with carrier sodium hydroxide were dissolved in 0.2ml of 0.3M sodium phosphate pH 7.2 (BDH, Analar) and 0.3ml P.B.S.. 100ul of NDOG2 antibody at a concentration of 2.6mg/ml in P.B.S. were then added, followed by 100ul Chloramine T solution (2mg/ml in 0.3M sodium phosphate). The iodination was allowed to proceed for 5 minutes with intermittent mixing and the reaction halted by the addition of 100ul of saturated L-tyrosine in 0.3M sodium phosphate. 100ul of H.P.P.F. was added as a carrier and the whole separated on the sephadex column. 3ml of P.B.S. (the capacity of the column) was then added and the first P.B.S. is added and the next fraction containing  $^{123}\text{I}$  labelled NDOG2 ( $^{123}\text{I}$  NDOG2) collected.

Sephadex G50 excludes molecules with a molecular weight greater than 50,000; thus high molecular weight substances such as  $^{123}\text{I}$  NDOG2 pass rapidly down the column whereas those of low molecular weight (i.e.  $^{123}\text{I}$  tyrosine) pass more slowly and may be discarded with the column.

A 20-30% yield of radioactivity was obtained in the  $^{123}\text{I}$  NDOG2 fraction giving a specific activity of  $4-8\text{M}_1\text{C/mg}$  of NDOG2. The preparation was made up to a convenient injection volume with normal saline and filtered through

a 0.2uM filter into the injection vial and transferred to the Department of Organ Imaging at the Bristol Royal Infirmary.

#### 5.2.5 Quality Control

The percentage of free  $^{123}\text{I}$  in the preparation was assessed prior to injection by paper chromatography. An aliquot of the radiopharmaceutical was placed at the bottom of a 2 inch long strip of Whatmans 3 chromatography paper. This was suspended so that the base was in contact with a solution of trichloroacetic acid (T.C.A.) which will precipitate protein and only allow free  $^{123}\text{I}$  to travel to the top of the paper. The paper was cut in half and each counted; the percentage of free  $^{123}\text{I}$  in the preparations used in this study varied between 1 and 10%.

#### 5.2.6 Preparation of $^{99\text{m}}\text{Tc}$ Human Serum Albumin

This was prepared using the T.C.K.-2 kit for labelling human albumin (International C.I.S.).

#### 5.2.7 Equipment used and Data acquisition

Data was acquired by collecting images of the biodistribution of the radiopharmaceutical over a 20 hour period. Between 40-70mBq of  $^{123}\text{I}$  NDOG2 was injected intravenously and three sets of sequential images of head and neck, chest, abdomen and pelvis obtained at approximately 10 minutes (early) 4 hours (intermediate) and 20 hours (late) after injection. In the breast carcinoma group images of the femora were obtained in certain cases. The patient was asked to empty her bladder prior to imaging on each occasion. The equipment used was an International General Electric Maxicamera 400T and a link systems data processor; the data

was collected on a 64 x 64 matrix, with a total number of counts for each image between 300,000 and 600,000.

By computer aided subtraction of early from later images, an attempt was made to enhance the visualisation of areas of increased radioactivity. In order to minimise the generation of artefacts and to improve the accuracy of subtraction images, the patient was repositioned before each image by the use of radioactive skin markers placed over the anterior superior iliac crests (Granowska et al 1984). Account of the short radioactive half life of  $^{123}\text{I}$  and of the biological half life of the radiopharmaceutical, was taken by measuring the activity in serial serum samples taken at the time of each imaging sequence. The ratio between plasma radioactivity at 4 hour and 20 hour after injection was calculated relative to the initial (10 min) radioactivity by Dr P Jackson and Mr G Staddon, of the department of Medical Physics, Bristol General Hospital. These values were 0.79 and 0.48 respectively and were incorporated into the subtraction technique.

### 5.3 Hazards

#### 5.3.1 Allergic Reactions

A hypersensitivity reaction could occur from an allergy to either iodine or to the NDOG2 antibody. Patients were questioned about any known allergies prior to injection of  $^{123}\text{I}$  NDOG2. Skin testing was not performed because of the theoretical risk of sensitising the patient to mouse protein. One patient, with an unsuspected allergy to iodine, did develop a mild allergic skin reaction after oral potassium iodide which responded to oral antihistamine therapy. Imaging was not performed in this case.



As P.L.A.P. is present in the circulation there is a theoretical risk of antibody/antigen complex formation and subsequent serum sickness. Other workers (Bagshawe et al 1980, Goldenberg et al 1980 and Halsall et al 1981) using antibodies to circulating antigens have not reported any such reaction and none occurred during this study, probably because of the very small (260ug) amounts of antibody injected.

### 5.3.2 Radiation

$^{123}\text{I}$  is unlikely to prevent a significant radiation hazard because of its short half life (13 hours) and the low dosage injected. Although P.L.A.P. is expressed by fallopian tube, endometrial and cervical columnar epithelium, this should not pose any hazard as these tissues had been or were to be removed during primary surgery. Despite trace amounts of P.L.A.P. in normal lung and thymus no specific accumulation of activity was demonstrated in these tissues in any patient imaged. The risks of accumulation of free  $^{123}\text{I}$  in thyroid and in gut mucosa have already been discussed (5.2.1.).

### 5.3.3 Myeloma Virus

Mouse myeloma cell lines contain oncornaviruses (Types C and A) which would be removed by affinity chromatography and gel filtration. Moreover there is no evidence that these viruses react with human cells.

## 5.4 Results with ovarian tumours

### 5.4.1 The effect of iodination on the specific activity of the NDOG2 antibody

Iodination can result in the loss of some specific activity by an antibody. In order to assess this both iodinated and non-iodinated NDOG2 were compared in an enzyme immunoassay initially described by McLaughlin et al (1983) and discussed in Chapter 4. There was a small (less than 10%) loss of activity in the iodinated antibody (fig 5.1).

### 5.4.2 Demonstration of in vivo specificity

Because of their increased vascularity and the greater permeability of their capillaries, tumours will accumulate foreign proteins to a greater degree than normal organs. In order to demonstrate that the images obtained were not solely due to a blood pool effect, 70mBq of  $^{99m}\text{Tc}$  albumin was injected intravenously and sequential images of pelvis and abdomen obtained in 3 patients prior to the injection, 2 days later, of  $^{123}\text{I}$  NDOG2. In one patient no tumour could be demonstrated by either method or by conventional imaging techniques but in the other 2 minimal uptake by pelvic tumours could be seen with  $^{99m}\text{Tc}$  albumin and this was much less than with  $^{123}\text{I}$  NDOG2 (fig 5.2 a & b).

A further patient (No. 11) whose tumour did not express P.L.A.P. and who had residual tumour was imaged using  $^{123}\text{I}$  NDOG2. None of her known tumour deposits were visualised although the stomach was clearly visualised, due to uptake of free  $^{123}\text{I}$  by gastric mucosa (fig 5.3).

#### 5.4.3 Normal Images

R.I.S. was not performed, for ethical reasons, on patients who did not have actual or suspected cancer. Therefore, normal imaging criteria were inferred from:

- a) The images obtained from 2 patients (No. 7 and 19) who were in remission at the time of imaging as judged by clinical examination, U.S.S. and a second look laparoscopy (patient 7).
- b) The images of 2 patients (No. 4 and 17) who had positive pelvic images from a full bladder and a fibroid uterus respectively but who were otherwise well gave information about normal abdominal, chest, head and neck images.
- c) The images of 3 patients (C, E and G) with breast carcinomas, without any evidence of pelvic metastases showed normal pelvic images.

A normal image reflects the blood pool distribution, thus the major blood vessels, heart, liver and spleen are clearly shown (fig 5.4 A, B, C and D). On subsequent images there are only changes of degree in this distribution with additional minor activity in the skeleton.

#### 5.4.4 False Positive Results

The cells of the reticulo-endothelial system will break down the injected radiopharmaceutical resulting in the presence of free  $^{123}\text{I}$  in the circulation. This can be taken up by the thyroid gland and gut mucosa as well as being excreted

in urine, giving rise to potentially misleading areas of high activity. Methods of suppressing uptake of  $^{123}\text{I}$  by the thyroid gland have already been discussed (5.2.1.).

In one case (patient 6), who was imaged because of elevated serum P.L.A.P. levels following radiotherapy, high activity was noted in the right iliac fossa in the intermediate (Fig 5.5) but not the early or late images. This activity was ascribed to the transient accumulation of  $^{123}\text{I}$  by caecal mucosa. The absence of any tumour detectable by C.T., the presence of activity in only one image and the patients continued long term good health make a recurrence of her tumour highly unlikely. This patient's serum P.L.A.P. was elevated and was secondary to cigarette smoking, this phenomenon has been discussed earlier (section 4.4.5 e (ii) and 1.3.8. f). Caecal uptake of free  $^{123}\text{I}$  was seen in one other case (patient 15).

Intense uptake of  $^{123}\text{I}$  by gut mucosa produced an area of high activity outlining the stomach in patient 11 (fig 5.3). This patient's tumour did not express P.L.A.P. and non-specific uptake by tumour was excluded as there was no tumour in this region at laparotomy.

Overall potassium iodide failed to prevent mucosal uptake of  $^{123}\text{I}$  in 2 from 18 patients (11%) and potassium perchlorate in 1 from 9 patients (11%), identical failure rates.

$^{123}\text{I}$  is cleared predominantly by the renal tract, therefore any obstruction can lead to an area of high activity. The bladder presents the greatest difficulty in interpreting pelvic images, because unless it is virtually empty during each imaging not only may genuine metastases be masked, but also false positive results generated particularly if a subtraction technique is used.

In one case (patient 4) chronic retention of urine gave a clinical impression, supported by evidence from a barium enema and ultrasound scan of an ovarian tumour. Failure to completely empty the bladder on early scan resulted in a region of apparent increased radioactivity to be generated when this was subtracted from a slightly radioactive but still full bladder on the intermediate image (fig 5.6 a, b and c).

Subtraction techniques in themselves have a potential for generating artefacts. The method used in this study involved the subtraction of the blood pool (early) image from the later images and this involves accurate realignment of the patient for each image, aided by the positioning of radioactive skin markers over the anterior superior iliac spines (A.S.I.S.). However, particularly if the patient is obese, the skin can move relative to the A.S.I.S. and incorrect alignment can occur with resultant subtraction artefacts.

There was a positive image in one case (patient 17) due to a fibroid uterus (fig 5.7). This was due, either to uptake of  $^{123}\text{I}$  NDOG2 by endometrium or, more likely, to the blood pool.

#### 5.4.5 Images obtained in patients with actual or suspected ovarian tumours

A comparison between the results of R.I.S. and the operative distribution of tumour is summarised in Table 5.3. These 13 patients were imaged prior to or shortly following surgery and 5 patients in this group had a second imaging procedure performed as well (Table 5.4). A further 6 patients were imaged following a course of chemotherapy or radiotherapy or when a change of chemotherapy was considered and the findings compared with either C.T. or U.S.S.. A final patient (No. 15) was imaged 6 weeks after macroscopic clearance of a stage 1c ovarian adenocarcinoma. This patient's serum P.L.A.P. levels were noted to have risen and her recurrent tumour was visualised both by C.T. and R.I.S..

Two groups of abnormal features were seen on the pelvic and abdominal images:

##### a) Focal abnormalities

These were seen in 12 patients, being most commonly present in the pelvis and numerous in one instance (patient 12). Subtraction was not usually necessary to demonstrate tumour (5.8) but in one instance the visualisation of the abnormality was enhanced by this technique (5.9 a, b and c). These abnormalities correlated well with surgical, U.S.S. or C.T. findings (Tables 5.3 and 5.4). A diffuse abnormality was also seen in two instances (patients 8 and 14). A focal lesion due to the probable blood pool image of a fibroid uterus had already been referred to in the previous section.



### b) Diffuse abnormalities

These were present in 3 patients and two of these also had a focal abnormality. In one case (patient 14) who had extensive intraperitoneal seedlings, probably in association with some ascites the diffuse activity was intense enough to mask the iliac vessels (fig 5.10).

The patients imaged with actual or suspected ovarian tumours are discussed in more detail below:

#### Patient 1

This 19 year old girl presented with what proved to be a stage 3 serous cystadenocarcinoma, which showed grade 4 reactive staining with NDOG2. Some residual tumour, which had spread directly from the primary, was known to be left in the pelvis after operation. She was followed up by serial serum P.L.A.P. estimations (patient 6, chapter 4) and was imaged after 4 courses of cisplatinum, to which she had failed to respond. A pelvic mass was visualised at R.I.S. and confirmed by U.S.S.. No other lesion was demonstrable by either imaging technique.

#### Patient 2

A 76 year old lady who was imaged prior to surgery for a stage 3 serous cystadenocarcinoma. The primary ovarian tumour and the single, large omental secondary were clearly visualised by R.I.S.. Both tumours showed grade 2 reactive staining with NDOG2. This patient declined further follow up.

### Patient 3

This 59 year old lady was imaged 8 days after surgery for a stage 3 serous cystadenocarcinoma. The tumour showed grade 3 reactive staining with NDOG2. Residual pelvic tumour was visualised by R.I.S. but secondary seedling deposits on colon and involved para-aortic nodes were not. C.T. failed to demonstrate any tumour deposits in this case. This patient has been followed up with serial serum P.L.A.P. estimations (patient 10 in chapter 4) and is at present in remission.

### Patient 4

This 70 year old lady was admitted in sub-acute obstruction. A mass was noted to be arising from the pelvis on abdominal examination. A barium enema showed extensive bowel compression and U.S.S. suggested the presence of a pelvic tumour. No preoperative serum P.L.A.P. levels were available, and the patient's obstruction settled after an enema. The 10 minute pelvic image showed a central cold area (fig 5.6 a) which at 4 hours appeared more active than the surrounding tissues (fig 5.6 b). Subtraction revealed an intensely active area (fig 5.6 c) which proved to be bladder. This patient did not have a pelvic tumour at E.U.A. and laparoscopy. Her symptoms and signs were due to constipation and chronic retention of urine.

### Patient 5

This 61 year old lady underwent a laparotomy one week prior to imaging. A large right sided serous cystadenocarcinoma with multiple intraperitoneal seedlings, ascites and a liver secondary were noted. Total abdominal hysterectomy with bilateral salpingo-oophorectomy and omentectomy was

performed. This tumour showed grade 4 reactive staining with NDOG2 and a diffuse abdomino-pelvic abnormality demonstrated at R.I.S.. The liver secondary was not visualised but the clearance time of the radiopharmaceutical from the liver was over twice that for the mean for this group of patients. This patient died within 2 months of surgery.

#### Patient 6

This 48 year old lady was imaged 21 months after primary surgery and subsequent radiotherapy for a stage 1a (ii) serous cystadenocarcinoma of the ovary. This tumour had shown grade 3 reactive staining with NDOG2 and serum P.L.A.P. levels were noted to be elevated. R.I.S. showed an area of increased activity in the right iliac fossa at 4 hours (fig 5.3) but not at 20 hours. A C.T. scan was normal and the patient remains well (patient 18 chapter 4). Her elevated P.L.A.P. levels were secondary to cigarette smoking and the abnormality demonstrated at R.I.S. was probably due to caecal uptake of free  $^{123}\text{I}$ .

#### Patient 7

This 33 year old patient had a laparotomy performed for a stage 3 serous cystadenocarcinoma. The primary and secondary tumours showed respectively grade 4 and grade 2 reactive staining with NDOG2. Residual intraperitoneal seedlings remained after surgery and the patient was imaged after a course of chemotherapy when in apparent remission. No abnormality was demonstrated at R.I.S. and a second look laparoscopy showed no bulk disease but positive washings were obtained. The patient (No. 19 in chapter 4) ultimately died of her disease.

Patient 8

This 74 year old patient was left with residual disease on bowel, bladder surface and liver edge after primary surgery for a grade 3 staining serous cystadenocarcinoma. R.I.S. was performed 2 months after surgery and following 2 courses of treosulphan. Focal pelvic lesions were demonstrated as well as diffuse abdomino-pelvic activity. The pelvic tumours were demonstrated by U.S.S. but the abdominal deposits were not.

Patient 9

This 53 year old lady presented with a pelvic mass 3½ years after total abdominal hysterectomy and bilateral salpingo-oophorectomy for menorrhagia. At that time a surprise histological diagnosis of a stage 1b serous cystadenocarcinoma was made. Unaccountably this patient received no further therapy and presented with a pelvic mass.

Laparotomy revealed a plaque of tumour adherent to the pelvic sidewall and surrounding the sigmoid colon, intra-abdominal seedlings and omental secondaries. Omentectomy was performed; the tumour showed grade 1 reactive staining with NDOG2. Serum P.L.A.P. was elevated (patient 22 in chapter 4) and R.I.S. performed 2 weeks later visualised the pelvic mass.

Patient 10

This 46 year old patient underwent a laparotomy which showed a serous cystadenocarcinoma of the ovary with peritoneal and omental secondaries. Biopsy alone was performed. Immunohistology of a paraffin section of this showed grade 1 reactive staining with NDOG2; however, as the dewaxing of paraffin sections causes denaturing of some of the NDOG2

determinant it was likely that this tumour expressed P.L.A.P. more strongly. This was born out by this patient's high serum P.L.A.P. levels (patient 25 in chapter 4). R.I.S. was performed 2 months after surgery following 1 pulse of chemotherapy and demonstrated the abdominal and pelvic tumour deposits. These were enhanced by subtraction (fig 5.8 a, b and c) and were also visualised by C.T..

#### Patient 11

This 22 year old patient with a stage 3 serous cystadenocarcinoma was imaged 2 months after primary surgery. There were residual intra-abdominal and pelvic tumour deposits although none were noted in the region of the stomach. Imaging showed a stomach shaped area of activity in the upper abdomen alone, thought to be due to  $^{123}\text{I}$  uptake by the gastric mucosa (fig 5.5). This patient's serum P.L.A.P. levels were normal, even in the presence of bulk disease (patient 31 in chapter 4).

#### Patient 12

This 25 year old patient was imaged 1 month after primary surgery for a stage 3 serous cystadenocarcinoma, which showed grade 4 reactive staining with NDOG2. Residual tumour on the rectum was demonstrated by R.I.S.

#### Patient 13

This 36 year old patient underwent total abdominal hysterectomy, bilateral salpingo-oophorectomy and omentectomy for an anaplastic stage 2b serous cystadenocarcinoma. Macroscopic tumour clearance was obtained but pelvic wall

peritoneal biopsies showed tumour involvement. The tumour showed grade 2 reactive staining and R.I.S. performed 2 months later after 1 course of chemotherapy showed recurrent pelvic tumour confirmed by C.T.. In addition an area of increased activity was demonstrated in the upper abdomen, possibly due to activity in stomach mucosa or to a liver secondary. C.T. of this area failed to detect any abnormality. This patient (No. 35 in chapter 4) continued to deteriorate despite chemotherapy.

#### Patient 14

This 73 year old patient was found, at laparotomy, to have massive intra-abdominal tumour secondaries arising from an ovarian adenocarcinoma. This tumour was biopsied only and showed grade 2 reactive staining with NDOG2. Imaging 3 weeks after operation and prior to chemotherapy, showed both diffuse and focal activity corresponding to metastases and primary tumours respectively. This patient was followed up with serial serum P.L.A.P. estimations (patient 43 in chapter 4).

#### Patient 15

This 43 year old patient underwent laparotomy with macroscopic clearance of her stage 1c poorly differentiated ovarian adenocarcinoma. She presented 6 weeks later with abdominal pain and elevated serum P.L.A.P. (patient 44 in chapter 4). R.I.S. demonstrated a pelvic recurrence (confirmed by C.T.) as well as an area of increased activity in the right iliac fossa corresponding to the caecum.



Patient 16

This 60 year old patient was imaged 8 months after surgery and following a course of chemotherapy for a stage 3 ovarian adenocarcinoma. Focal pelvic lesions were demonstrated by R.I.S. and confirmed by U.S.S. following elevated serum P.L.A.P. (patient 15 in chapter 4).

Patient 17

This 62 year old patient presented with a pelvic mass and was imaged preoperatively, without knowing serum P.L.A.P. levels. A focal abnormality (fig 5.7) was demonstrated in the pelvis and at operation this was found to be a fibroid uterus. The image obtained was due either to the blood pool within the enlarged uterus or due to uptake by endometrium. This patient's P.L.A.P. levels were later noted to be normal.

Patient 18

This 62 year old patient was imaged 4 weeks after surgery for a stage 3 serous cystadenocarcinoma of the ovary. This tumour showed grade 4 reactive staining. Residual pelvic tumour was demonstrated, by R.I.S. but intra-abdominal peritoneal seedlings were not visualised by this technique or U.S.S.. This patient (No 32 in chapter 4) has been followed up with serial serum P.L.A.P. estimations, and her subsequent deterioration predicted by assay 3.

#### Patient 19

This 44 year old patient was imaged after a 6 month course of chemotherapy following total abdominal hysterectomy and omentectomy for a stage 3 serous cystadenocarcinoma. Small (less than 5mm diameter) deposits were left after surgery and her tumour showed grade 4 reactive staining with NDOG2. No abnormality was demonstrated by either R.I.S. or U.S.S. and the patient remains well with normal serum P.L.A.P. levels (patient 24 in chapter 4).

#### Patient 20

This 49 year old patient was imaged after four pulses of adjuvant chemotherapy following macroscopic clearance of a stage 3 ovarian adenocarcinoma. Serum P.L.A.P. was noted to be elevated (patient 14 in chapter 4) and R.I.S. showed a focal pelvic lesion that was also visualised by U.S.S..

#### 5.4.6 Demonstration of antibody on resected tumour after R.I.S.

Frozen sections of tumour, resected 24 hours after the injection of  $^{123}\text{I}$  NDOG2, of 6u thickness were cut, air dried and fixed in acetone. They were incubated for 30 minutes with peroxidase conjugated rabbit anti mouse immunoglobulin and then with  $\text{H}_2\text{O}_2$  DAB (chapter 3). Positive reactive staining was demonstrated on the resected tumour (fig 5.11) demonstrating the presence of NDOG2.

#### 5.4.7 Comparison with other imaging techniques

In 12 patients with actual or suspected ovarian cancer C.T. or U.S.S. was performed within 1 week of R.I.S. and the results compared (Table 5.4). In 5 of these patients the extent of the tumour had been mapped out by recent surgery (patients 3, 4, 8, 10 and 13).

No tumour deposit detectable by C.T. or U.S.S. was missed by R.I.S. and in one instance (patient 3) R.I.S. detected a focal tumour deposit that was not visualised by C.T.. In addition there were seedling tumour deposits on the surface of the colon and spread to the para-aortic nodes. Neither of these sites of metastasis were detected by either imaging technique. In a further case (patient 8) although both U.S.S. and R.I.S. detected a pelvic tumour only R.I.S. identified this patient's widespread peritoneal seedlings. In two cases (patients 7 and 19) neither imaging technique detected any tumour. One of these (patient 7) positive peritoneal washings only at second look laparoscopy. Both patients were clinically in remission at the time of imaging although patient 7 subsequently developed intra-abdominal secondary tumour and died.

A chronically distended bladder gave a false positive image (patient 4) but interestingly both barium enema and an U.S.S. suggested the possible presence of an ovarian tumour.

In two patients (No. 6 and 15) an area of increased activity was noted in the right iliac fossa at 4 hours in both cases. C.T. did not show a tumour in this region, although patient 15 did have a pelvic recurrence of her tumour from which she rapidly died, despite chemotherapy. The area of increased activity disappeared on the 20 hour scan in patient 6, who remains well some 11 months later and it is unlikely that this represented anything other than uptake by caecal mucosa of free  $^{123}\text{I}$ .

### 5.5 Results of R.I.S. performed on patients with breast, endometrial and testicular tumours

5 patients with metastatic breast cancer, all of whom had the primary tumour resected, 1 patient with a stage 4 endometrial carcinoma and 1 patient with a disseminated seminoma were all imaged using an identical imaging technique to that used for patients with ovarian cancer.

The results of imaging in this group of patients is summarised in Table 5.5.

#### Patient A

This 45 year old man, who was a heavy cigarette smoker, developed mediastinal, anterior chest wall and neck metastases from his seminoma. His serum P.L.A.P. levels were elevated but none of his known metastases were visible at imaging.

#### Patient B

This 64 year old lady, who did not smoke, had a stage 4 endometrial carcinoma (invading the bladder but with no obvious distant metastases) which showed grade 3 reactive staining with NDOG2. Serum P.L.A.P. was elevated and imaging demonstrated a large pelvic tumour.

#### Patient C

This 70 year old patient had lumbar spine metastases of her breast carcinoma which were not visualised at R.I.S..

Patient D

This 55 year old lady developed a metastasis in her right lung 4 years after a right mastectomy. This metastasis was clearly visualised on both the 4 hour and 20 hour image, being most clearly visualised on the latter.

Patient E

This 82 year old patient had both right femoral and left neck nodal breast metastases which were not visible at imaging.

Patient F

This patient aged 64, had widely disseminated tumour present in both lumbar and sacral spine and right hip. Only her lumbar metastasis was visualised at R.I.S..

Patient G

This patient with skull and left chest wall breast metastases was imaged, but neither tumour deposit was visualised.

Overall, imaging patients with breast cancer using  $^{123}\text{I}$  NDOG2 proved disappointing, with only 2 from 9 known sites of secondary tumour being visualised. None of the sites of metastatic seminoma were visualised. This patient's high serum levels of P.L.A.P. could well have been secondary to cigarette smoking. It is also possible that this tumour expressed a form of P.L.A.P. not recognised by NDOG2. Unfortunately, no fresh tumour tissue was available from this patient.

### 5.6 Dosimetry

The dosimetry for this antibody was calculated by Dr P Jackson of the Department of Medical Physics, Bristol General Hospital (Jackson et al 1985).

$^{123}\text{I}$  NDOG2 was cleared from the blood pool by the redistribution of the radiopharmaceutical into other compartments and the excretion of free  $^{123}\text{I}$  by the kidneys. The mean plasma clearance time of the compound was 20.8 hours and 79% of the injected activity (correcting for radioactive decay) was present in urine after 48 hours. The amount of free  $^{123}\text{I}$  present in serum, at any one time, was low and varied between 1.3% and 7.3% (mean 3.7%). The tumour to background (thigh) ratios varied from 1.4:1 and 4.8:1 (mean 2.95:1) and these figures are comparable to other studies (Goldenberg et al 1980, Kim et al 1980, Bagshawe et al 1980).

The whole body dose of radioactivity received by the patient was 60mrem/mCi (16.3uSv/mBq).

### 5.7 Conclusions and Discussions

This chapter describes the preparation of radiolabelled NDOG2 and its use in a variety of tumours, predominantly ovarian carcinomas.  $^{123}\text{I}$  was used as the radiolabel in this study as it is a pure gamma emitter, has an energy range ideally suited to the modern gamma camera and can be easily linked to proteins. However, it has a short half life and is only produced weekly but is considerably cheaper than  $^{111}\text{In}$ . Uptake of free  $^{123}\text{I}$  by gut mucosa and



thyroid can, in theory, be blocked using potassium iodide or perchlorate. In this study potassium iodide proved to be the more effective thyroid blocking agent although neither compound totally prevented uptake by gut mucosa. This latter was a significant cause of false positive images.

The chloramine T method was used to iodinate NDOG2 and this is a simple technique and  $^{123}\text{I}$  NDOG2 retains upwards of 90% of the specific activity of the unlabelled form.

There is a theoretical risk of serum sickness when an antibody directed against a circulating antigen is injected intravenously. Only a small quantity (260ug) of antibody was used in this study and no such reactions were seen. Indeed other workers have used up to 20mg of antibody without ill effect (S. Larson - personal communication). No animal models were available but the *in vivo* specificity of  $^{123}\text{I}$  NDOG2 has been demonstrated in humans (section 5.4.2.).

Two forms of abnormality due to ovarian cancer were demonstrated with R.I.S. using NDOG2, focal and diffuse, and these have been described already (section 5.4.5). The R.I.S. findings correlated well with images obtained at U.S.S. or C.T. and with the operative findings. However, in certain situations it was difficult to visualise ovarian tumour deposits particularly when they are in, or close to, areas of high background activity i.e. liver and the para-aortic region. This is due to a high proportion of the injected activity remaining in the circulation and this

can mask specific tumour uptake. Overall tumour to background ratios were low (mean 2.95:1) and so methods of subtracting the early from later images were explored to try and eliminate the background activity. However, even when the biological half life of  $^{123}\text{I}$  NDOG2 was taken into account this method did create artefacts. It proved impossible to be completely accurate in repositioning the patient prior to each imaging sequence and so the early and later images were not always perfectly superimposed.

Other causes of areas of high activity, not due to tumour uptake of the radiopharmaceutical were the accumulation of  $^{123}\text{I}$  in gut mucosa and bladder. Potassium iodide and perchlorate never proved totally effective in eliminating this and in one case (patient 6) caecal uptake of  $^{123}\text{I}$  at 4 hours and raised serum P.L.A.P. (due to cigarette smoking) raised the suspicion of a tumour recurrence. However, a normal C.T. scan and the transient nature of the uptake suggested otherwise. The patient has subsequently remained well with no evidence of tumour recurrence.

It is vital that the bladder is completely empty before imaging the pelvis as in one case (patient 4) chronic retention of urine gave a clinical and R.I.S. impression of a tumour. Conversely a full bladder may also mask specific uptake of  $^{123}\text{I}$  NDOG2 by a pelvic tumour deposit.

The results of imaging patients with breast cancer or seminoma were disappointing. In the latter case the patient's elevated serum P.L.A.P. was most likely due to cigarette smoking. Only 2 from 9 known sites of breast metastasis were detected by this method; other workers (McDicken et al 1983) have reported reactive staining with the H317 anti P.L.A.P. antibody but have failed to detect raised serum levels in these patients. As no fresh tumour was available from patients with breast cancer who were imaged it is possible that many did not express P.L.A.P..

## 5.7 Tables, Charts and Figures

### Legends for figures

- 5.1 Comparison of NDOG2 and  $^{123}\text{I}$  NDOG2 specific binding capacities using assay 1.
- 5.2
  - a) Pelvic image using  $^{99\text{m}}\text{Tc}$  human serum albumin at 4 hours.
  - b) Pelvic image in the same patient using  $^{123}\text{I}$  NDOG2 at 4 hours.
- 5.3 Abdominal image showing stomach mucosal uptake of  $^{123}\text{I}$ .
- 5.4 Normal late images of
  - a) Pelvis
  - b) Abdomen
  - c) Chest
  - d) Head and Neck.
- 5.5 Pelvic image at 4 hours showing caecal uptake of  $^{123}\text{I}$ .
- 5.6 Pelvic images, to demonstrate bladder artefact generated by subtraction at
  - a) 10 minutes
  - b) 4 hours
  - c) subtracted image.
- 5.7 Intermediate pelvic image of a patient with a fibroid uterus.
- 5.8 Late pelvic image demonstrating a focal lesion.

- 5.9 Series of pelvic images, to demonstrate enhanced visualisation of tumour by subtraction, at:
  - a) 10 minutes
  - b) 20 hours
  - c) subtracted image.
- 5.10 Late abdominal image showing a diffuse abnormality.
- 5.11 Demonstration of NDOG2 on resected tumour.

FIG 5.2 A



FIG 5.2 B



FIG 5.3

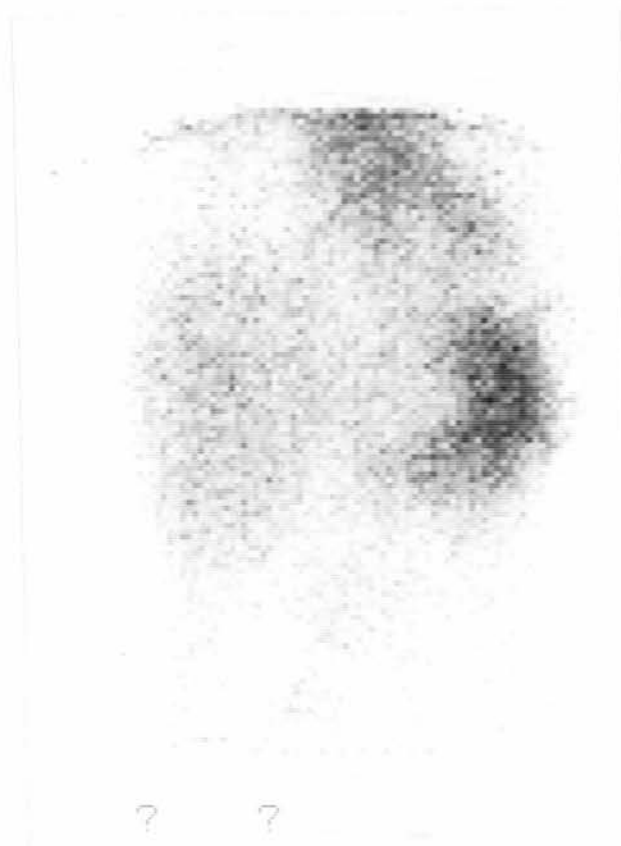




FIG 5.4 A

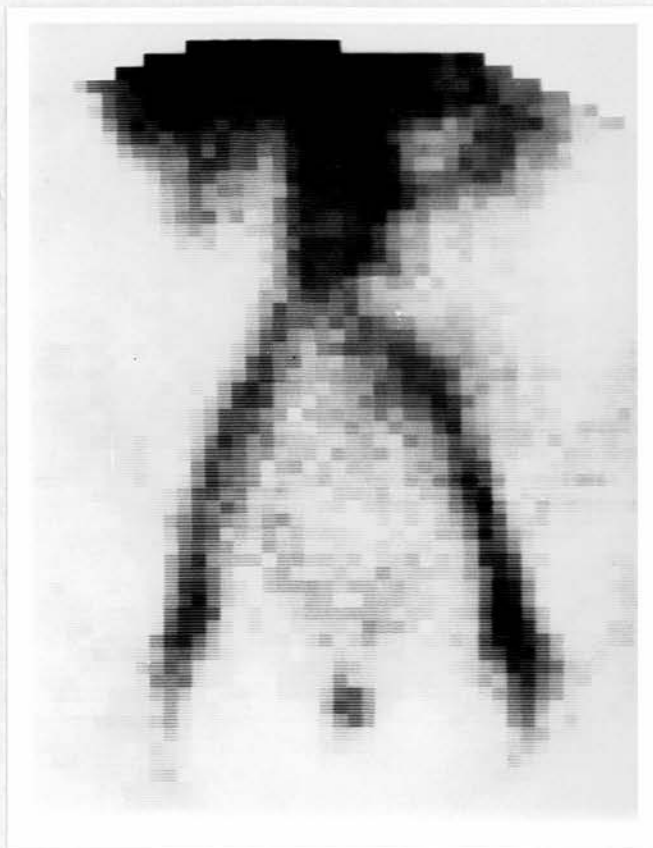


FIG 5.4 B

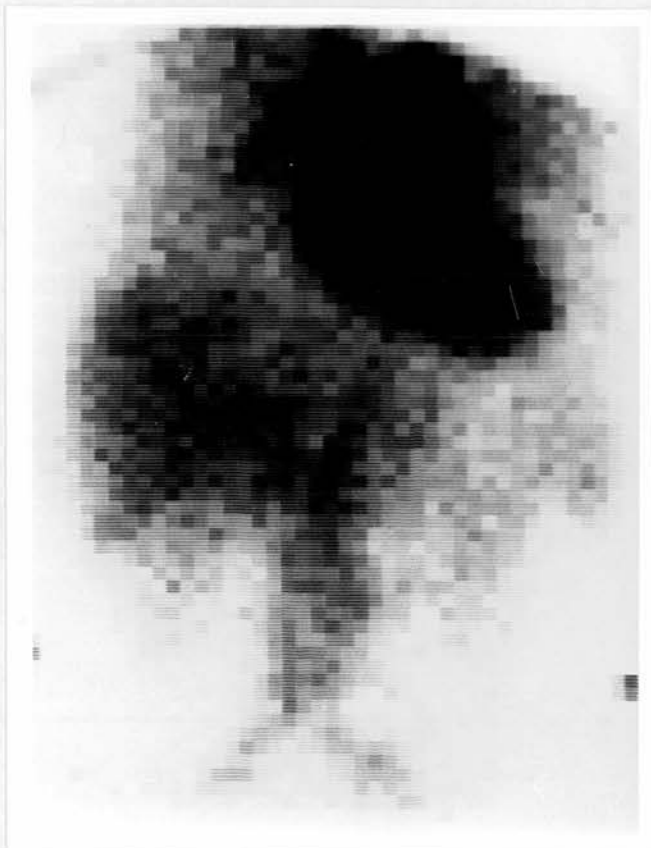


FIG 5.4 C

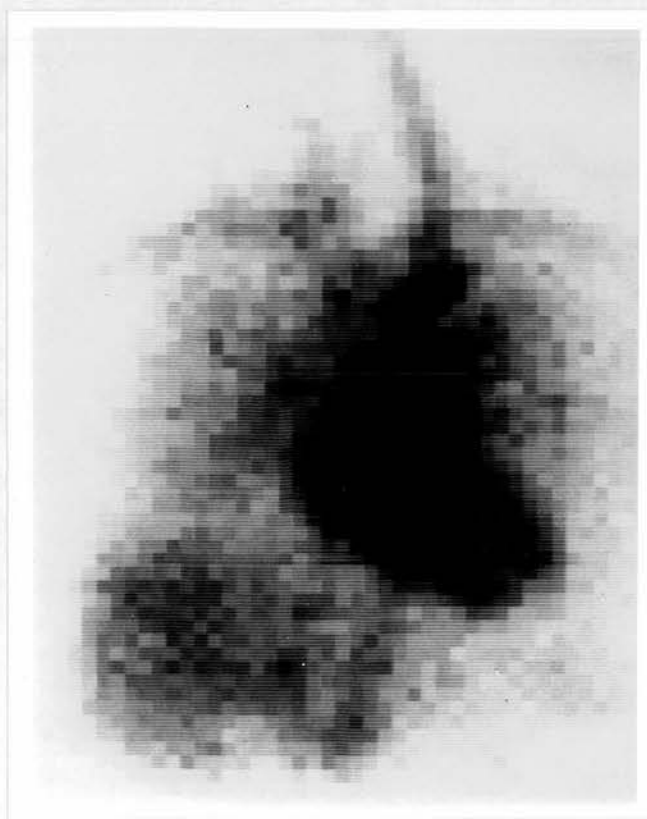


FIG 5.4 D



FIG 5.5



FIG 5.6 A



FIG 5.6 B



FIG 5.6 C

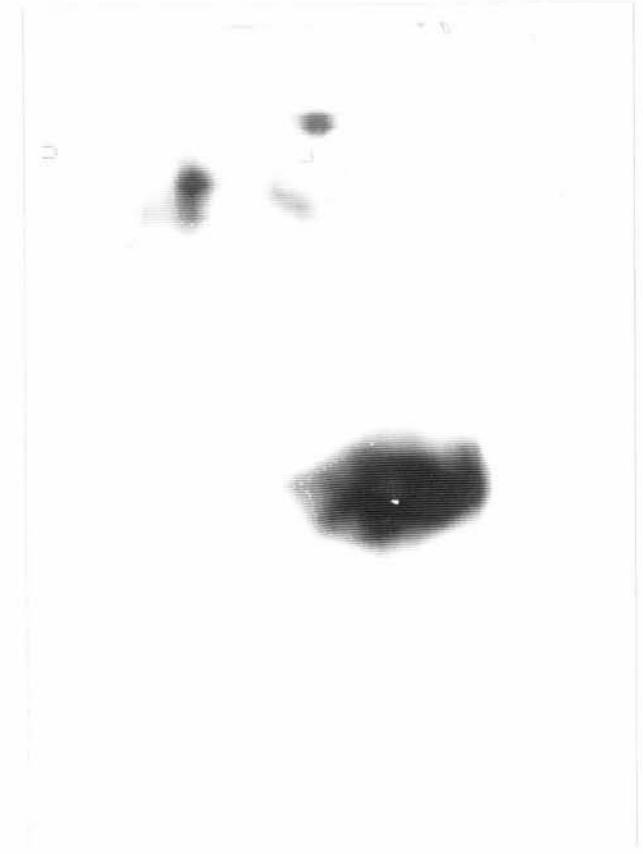


FIG 5. 7

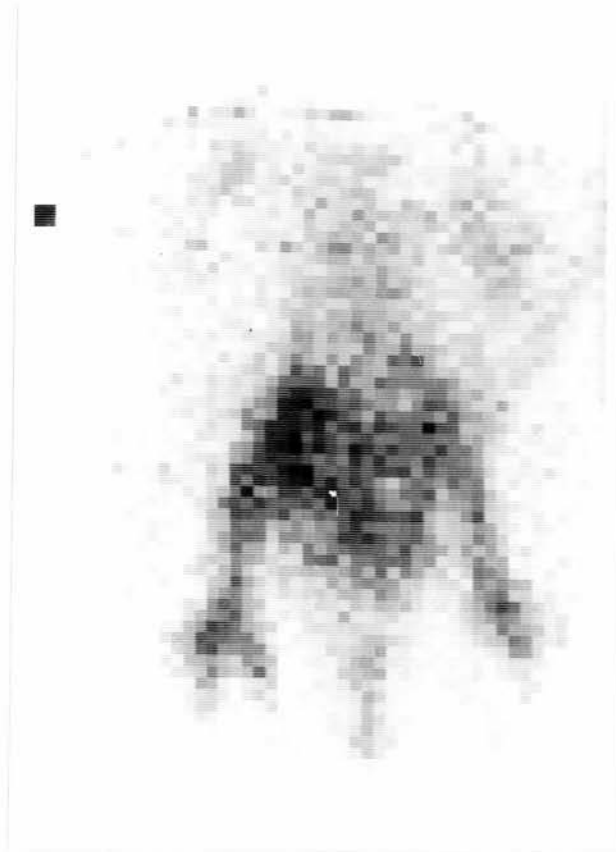


FIG 5.8

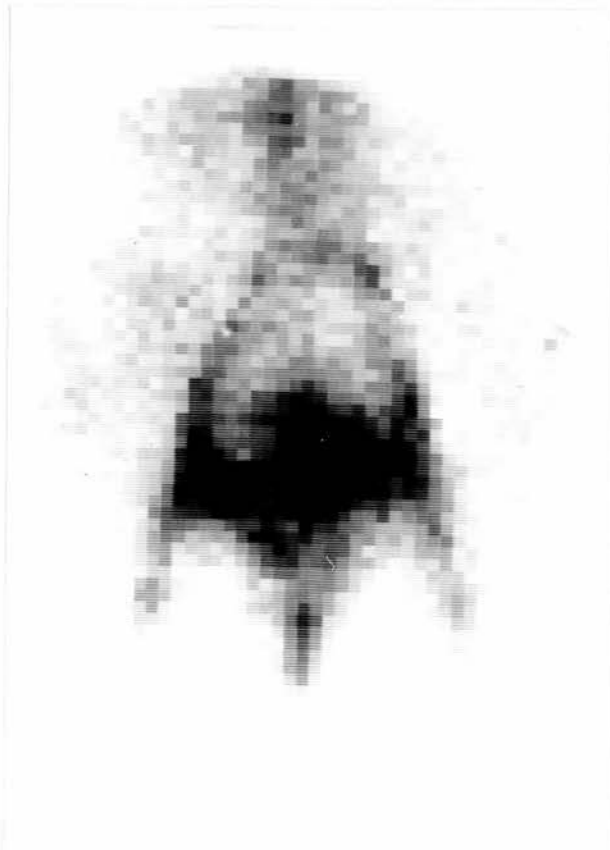


FIG 5.9 A



FIG 5.9 B

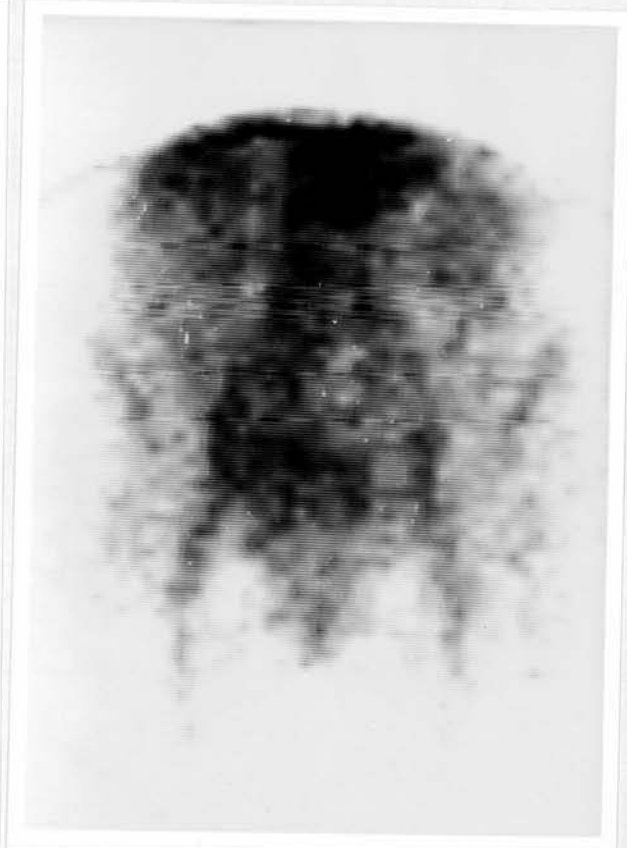


FIG 5.9 C



FIG 5.10



FIG 5. 11

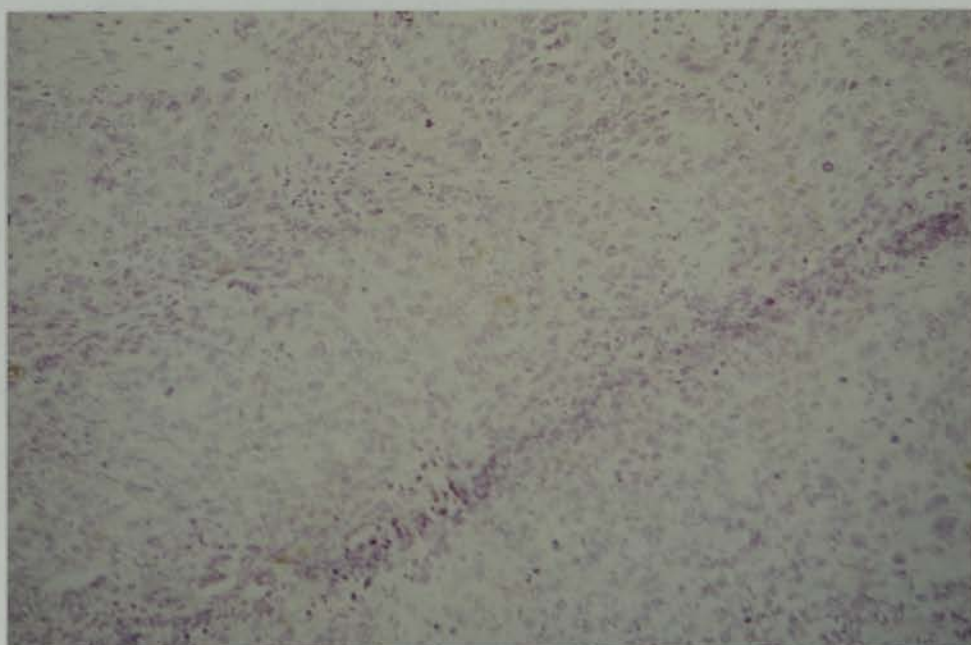


Fig 5.1 Comparison of the specific activities of NDOG2 and  $^{123}\text{I}$  NDOG2

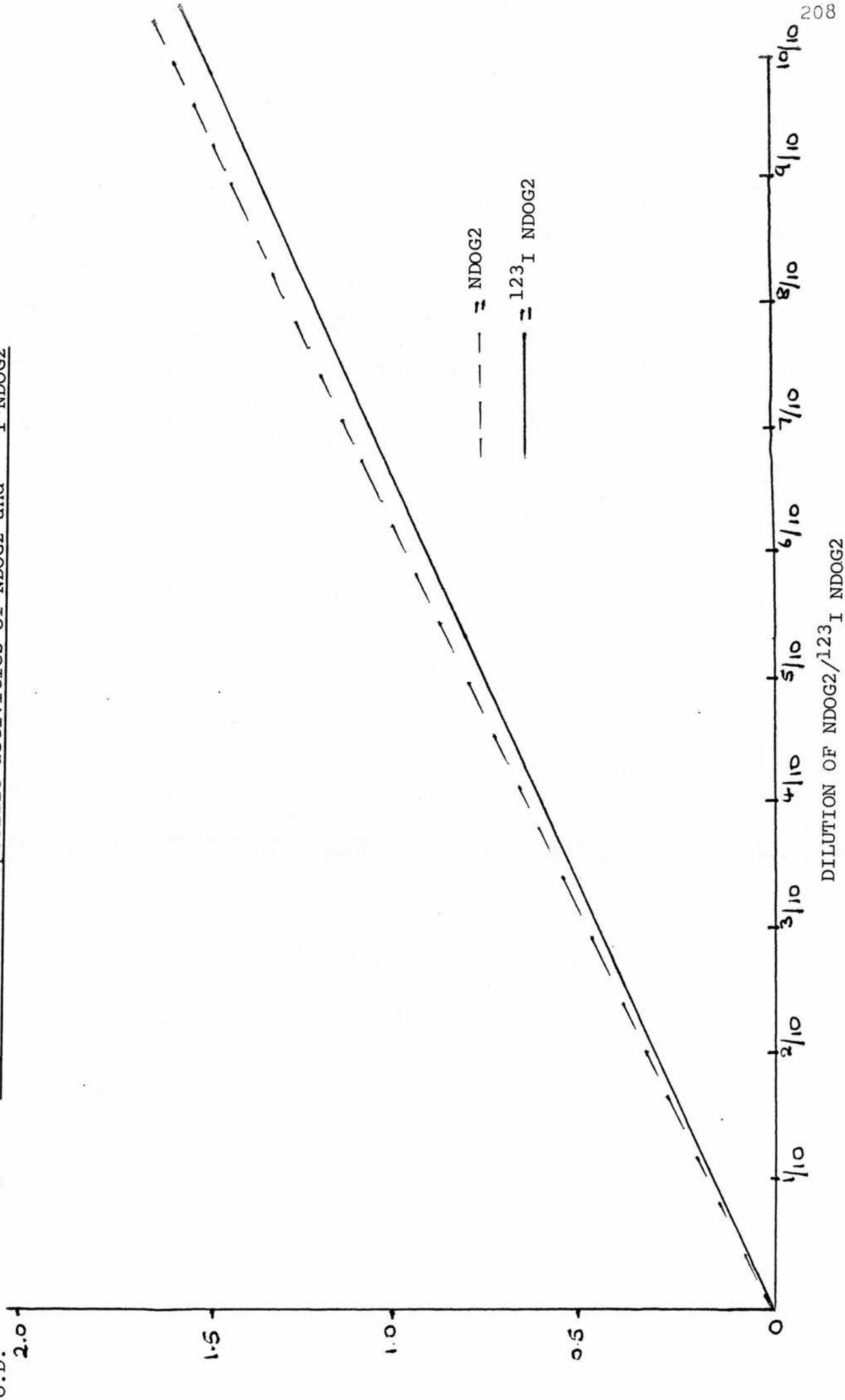


Table 5.1 Selection criteria of patients with ovarian cancer for R.I.S.

Patient	Positive Immunohistology alone	Elevated serum P.L.A.P. alone	Both	Non P.L.A.P. producing tumour	P.L.A.P. status unknown prior to R.I.S.
1			✓		
2					✓
3	✓				
4					✓
5	✓				
6			✓		
7	✓				
8	✓				
9			✓		
10		✓			
11				✓	
12	✓				
13			✓		
14			✓		
15		✓			
16		✓			
17					✓
18			✓		
19	✓				
20		✓			



Table 5.2     Comparison of results with  $^{123}\text{I}$  NDOG2 and  $^{99\text{m}}\text{Tc}$  H.S.A.

Patient	Site of tumour	$^{123}\text{I}$ NDOG2	$^{99\text{m}}\text{Tc}$ H.S.A.
18	a) Pelvic Residual Tumour	+	±
	b) Intraperitoneal Seedlings	-	-
19	In remission clinically	-	-
20	Pelvic mass	+	±

Table 5.3 Comparison of operative and radioimmunoscintigraphy findings in ovarian carcinoma

Patient	Operative Findings	R.I.S. findings
2	a) Ovarian Primary b) Omental Secondary	+ (focal) + (focal)
3	a) Pelvic residual disease b) Colonic seedling c) Para-aortic nodes involved	+ (focal) - -
4	Chronically distended bladder	false positive
5	a) Liver metastasis b) Ascites c) Peritoneal seedlings 5mm (numerous)	- + (diffuse)
8	a) Residual bowel seedlings b) Metastases on bladder surface	+ (diffuse) + (focal)
9	a) Residual pelvic sidewall (left) disease b) Peritoneal seedlings	+ (focal) -
10	a) Ovarian primary b) Omental secondaries	+ (focal) + (focal)
11	Residual intraperitoneal tumour deposits	- (uptake by stomach)
12	Residual deposits on rectum	+ (focal)
13	a) Residual microscopic deposits on right pelvic side wall b) No obvious liver metastasis	a) + (focal) b) Focal in liver
14	a) Left ovarian primary b) Omental secondary c) Intraperitoneal seedlings (numerous)	a) + (focal) Diffuse Diffuse
17	Fibroid uterus	+ outlining uterine cavity
18	a) Residual pelvic disease b) Intraperitoneal seedlings	+ (focal) -

Table 5.4 Comparison of radioimmunoscintigraphy and  
U.S.S. or C.T. findings in ovarian carcinoma

Patient	C.T. or U.S.S.	R.I.S.
6	No tumour (C.T.)	Uptake by caecum at 4 hours. None at 20 hours
7	No tumour (U.S.S.)	Normal
16	Pelvic mass (C.T.)	+ (focal)
15	Pelvic mass (C.T.)	+ (focal) and uptake by caecum at 4 hours
19	No tumour (U.S.S.)	Normal
20	Pelvic mass (U.S.S.)	+ (focal)
1	Pelvic mass (U.S.S.)	+ (focal)
* 3	No recurrence (C.T.)	+ (focal) Pelvic sidewall
* 4	Extrinsic compression of rectum (Barium enema) Possible pelvic tumour (U.S.S.)	False positive
* 8	Pelvic mass (U.S.S.)	+ (focal) and Diffuse
* 10	Pelvic mass (C.T.) Abdominal mass (C.T.)	+ (focal) + (focal)
* 13	Pelvic mass (C.T.)	+ (focal) + (liver)

\* Recent laparotomy

Table 5.5     The results of R.I.S. in patients with breast, testicular and endometrial cancers

Patient	Tumour	Sites of known tumour	R.I.S.
A	Seminoma	a) Mediastinum b) Chest wall c) Neck	- -
B	Endometrium	Uterus with extension to pelvic side wall and bladder	+ve (focal) pelvic tumour
C	Breast	Lumbar spine metastases	-
D	Breast	Lung	+ve (focal)
E	Breast	a) Neck lymph nodes (L) b) Femur (R)	- -
F	Breast	a) Lumbar spine b) Sacrum c) Right hip	+ve (focal) - -
G	Breast	a) Skull b) Left chest wall	- -

CHAPTER 6

## SYNOPSIS

The suggestion that NDOG2 fixes a P.L.A.P. determinant arose from the study of normal placentae of varying gestational ages. The NDOG2 determinant first appeared on placentae at 10 weeks gestation and was present on all placentae examined from 16 weeks to term. This is in line with the autogeny of P.L.A.P.. The further experimental work performed to show that the placental isoenzyme alone is recognised has been described in sections 2.3 and 3.3.1. Dr J Millan of Umea University, Stockholm, independently confirmed these findings and showed that NDOG2 reacted with the three common allelic forms (F, I and S) of P.L.A.P..

The possibility that NDOG2 recognises something other than enzymically active P.L.A.P. came from the comparison of the results obtained with assays 1 and 3. The first assay measured active enzyme only whereas assay 3 measured the total protein captured by NDOG2. There were many similarities between the two assays (section 4.7) but the most striking difference was noted in the wide range of results obtained in the normal population using assay 3. An attempt was made to identify this antigen by affinity chromatography and immunoblotting (section 4.8). A band, at 67,000 Daltons was identified in 'high normal' and malignancy sera and in addition several lower molecular weight proteins were detected in the sigma P.L.A.P. preparation, using the polyclonal preparation, in the blotting experiments. However, the second antigen was not clearly identified. This proposed second antigen would need to have one determinant recognised by NDOG2 and at least one other recognised by the polyclonal antiserum, for assay 3 to work. Therefore assay 3 measures both enzymically active

P.L.A.P. and either an inactive form of this enzyme or a similar tumour associated antigen. The nature of this is uncertain but it may be a hybrid alkaline phosphatase molecule. Because the polyclonal antibody cross reacts with intestinal alkaline phosphatase (Dako Data Sheet), assay 3 could measure a hybrid consisting of one subunit P.L.A.P. and a second subunit of intestinal alkaline phosphatase. Such molecules have been described (Warnock and Reisman 1969, Higashino et al 1972).

Immunohistological examination of normal tissues were performed to assess the distribution of the NDOG2 determinant in health. This distribution corresponds to that reported for P.L.A.P. with the exception of testis. No reactive staining was seen with NDOG2 in this organ but the form of P.L.A.P. reported in testis was similar to the rare placental D variant (Goldstein et al 1982), a type not recognised by NDOG2.

Epithelial cancers of the ovary, cervix and endometrium all arise from structures that have a close embryological origin and all express P.L.A.P. to some degree. Cervical carcinomas showed the weakest degree of reactive staining with NDOG2 with only 16% showing greater than grade 1' staining, a figure similar to that reported in the literature for P.L.A.P. (Cadeau et al 1974). A much greater percentage (65%) of endometrial cancers show positive staining (58% greater than grade 1) which is higher than described by many workers for P.L.A.P. (Cadeau et al 1974, Van Nagell et al 1981 and Nozawa et al 1981) but is in accord with the findings of Doellgast and



Homesley (1984). Expression of the NDOG2 determinant by these tumours is not affected by their stage or differentiation as suggested by Kellen et al (1976) who found that only stage Ia tumours produced P.L.A.P.. Interestingly both samples of adenosquamous endometrial cancers showed no reactive staining.

Relatively effective methods exist to follow up patients with endometrial and cervical cancers, however, as had been discussed in chapter 1, the situation is different with ovarian cancer. This thesis has concentrated on methods, based on the NDOG2 antibody, to assess the response of a patient with ovarian cancer to therapy. Positive reactive staining with NDOG2 was found in 65% of 56 malignant ovarian tumours. This was noted predominantly in serous cystadenocarcinomas and serous cystadenomas. The degree of staining was not affected by the differentiation of the tumour but heterogeneity of expression was noted between primary and secondary tumours in the same patient. In general, secondary tumours expressed this antigen to a lesser degree than their primary and those cells that were positive were present in clumps suggesting an origin from a single cell (fig 3.9). This points to the presence of more than one cell line in these tumours. The distribution of the NDOG2 determinant has a very similar distribution, in both normal and malignant gynaecological organs, to that of another tumour associated antigen, CA 125 (Davies et al 1985). However, this latter does not fix a P.L.A.P. determinant (Bast et al 1985).

From the distribution of the NDOG2 determinant in both normal organs and ovarian cancer, P.L.A.P. would seem to be a suitable potential tumour marker for this disease. It is not truly tumour specific but a number of the normal tissues that express it are either irrelevant (placenta) or will usually have been removed at primary surgery (fallopian tube, endometrium and cervix). Expression by the thymus is minute which leaves the lung as the only significant source of this antigen in healthy tissue. Unfortunately this source proved to be a cause of false positive results in the serum assays as cigarette smoking, by a direct effect on lung, results in elevated serum P.L.A.P..

Two workable assays were used to follow up patients with ovarian cancer and of these assay 1 was the better understood. This assay measured active P.L.A.P. and a definite upper limit of normal was determined for this in the healthy, non-smoking population. Assay 3 was less satisfactory in that there was a huge variation of expression between healthy individuals. The significance of this has been discussed earlier and in section 4.6.4. e (ii). The lung was the most likely source of this antigen as individual variation in reactive staining was seen in the different samples examined.

Assay 3 proved the better assay in accurately predicting the course of the disease, but there were 2 cases where assay 2 proved superior (patients 22 and 23). In these cases both of the assays initially agreed with each other

and the course of the disease, but then assay 3 fell inappropriately. The reverse situation occurred in 3 cases (patients 12, 15 and 44), however, in these the rate of increase of assay 3 slowed after the fall in assay 1 (fig 4.7). This, I think, tends to suggest the presence of at least 2 separate cell lines in these cases, one expressing active P.L.A.P. and the other expressing the antigen referred to earlier.

Inappropriate falls in either or both assays were seen in a total of 10 patients. In all cases an initially increasing level reflecting tumour growth was followed by a fall, although the tumour continued to grow. This could be due to a changing cell population, perhaps reflecting different sensitivities to chemotherapy.

In general the immunohistology of the tumour gave a good guide as to who should be followed using these assays. There were however exceptions. In one case (patient 2) with a tumour of borderline malignancy, levels were never elevated although this tumour showed grade 3 reactive staining with NDOG2. This may be due to a property of such tumours and in this context it is interesting to note the normal levels of P.L.A.P. in preoperative serum samples of patients with benign ovarian tumours. Pre and post operative levels in assay 3 were the same in these cases but all of these tumours had positive reactive staining with NDOG2. In another 6 cases the immunohistology of the tumour was a poor guide. In 2 such cases (patients 19 and 34) the assays were of poorer predictive value than

might have been expected, possibly reflecting a changing cell population or the lower potential for metastasis of P.L.A.P. producing cells. The reverse situation occurred in 3 cases (patients 21, 30 and 36), perhaps reflecting unrepresentative tumour sampling as one or both assays accurately reflected tumour load from before operation to death.

R.I.S. was the other method of using NDOG2 to assess the extent of this disease. In patients with ovarian cancer, this technique appeared safe and at least as accurate as C.T. or U.S.S. in detecting tumour deposits. The success of R.I.S. depended firstly on the tumour expressing the NDOG2 determinant but also on a number of other factors. Those tumour deposits in or close to areas of high background activity, such as the para-aortic nodes and liver, were not visualised; however in the latter case a prolonged clearance time was noted. In order to remove this background activity a subtraction technique was performed as described in section 5.2.7. However, this did not improve the sensitivity of the technique although it did clarify areas of increased activity seen in the unsubtracted images. It frequently proved impossible to totally accurately reposition the patient for each set of images and thus subtraction artefacts were generated. Small intra-abdominal deposits were not visualised (e.g. patient 9) unless numerous.

There were a number of false positive results which have already been described (section 5.4.4), however two of these warrant further discussion. Uptake of free  $^{123}\text{I}$  by gut mucosa occurred in 3 cases and proved impossible to

stop regardless of which blocking agent was used. As ovarian cancer frequently metastasises to the surface of gut a situation could arise that would make the interpretation of some images difficult. A full bladder gave rise to a false positive result in one case (patient 4) but equally this could have masked a genuine deposit. It is essential to ensure that the bladder is definitely empty prior to each set of images.

R.I.S. with  $^{123}\text{I}$  NDOG2 was a poor imaging technique in the small number of patients imaged with disseminated breast cancer. This is a tumour that expresses P.L.A.P. but whose serum levels are often normal (McDicken et al 1983); in these cases there was no way of ensuring that the tumours did express the NDOG2 determinant, perhaps a reason for the disappointing results.

This thesis has described 2 methods of using the NDOG2 antibody to assess the response of a patient, with ovarian cancer, to therapy. Both methods have their share of false positive and false negative results but there are many advantages in using NDOG2 in a serum assay compared to its use in R.I.S.. The serum assays are less time consuming for both operator and patient, cheap and a large number of patients can be screened in a day. In addition there are no potential hazards (e.g. allergic reaction) to the patient. Admittedly the serum assay will not localise the position of the tumour recurrence, but in the case of ovarian cancer, this is largely irrelevant to the patient's prognosis.

However, R.I.S. does give information about the biodistribution and tumour uptake of the antibody in vivo, which is essential before antibody directed therapy can be considered. This has not been performed using NDOG2 but some workers have claimed at least short term success, using this treatment modality in ovarian cancer (Hammersmith Oncology Group 1984).

The NDOG2 antibody only recognises a subpopulation of cells in any one tumour and the response of this population to chemotherapy may not be representative of the tumour as a whole. Further work is being directed to the development of a panel of monoclonal antibodies in an attempt to identify the various cell lines present in ovarian cancer and to subsequently use these in antibodies serum assays to follow up patients with this disease.

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